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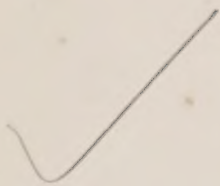
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A STUDY OF
Antimetabolites

A STUDY OF Antimetabolites

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for Medical Research



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To Dr. H. S. Gasser
whose searching criticisms have held
the wings of my imagination,
and to one
whose quiet listening has let it soar.

Preface

The purpose of this book is to present an idea, or more correctly, a group of closely related ideas. These have arisen during the past decade as a result of the finding of a number of experimental facts, all of which indicate that the biological functioning of diverse kinds of essential metabolic substances can be antagonized by the presence of other compounds closely related in chemical structure to them. From these facts have arisen an opinion, which is held in several quarters, that useful pharmacological agents may be realized by careful study of this phenomenon, and that a deeper insight into natural processes of the living world may be uncovered with them. Such ideas have been a powerful stimulant to experimentation in this field, and a considerable body of knowledge is being rapidly accumulated. It was felt that a useful purpose might be served if this factual information were assembled in as brief a form as is consistent with clarity, and also if attention were called to underlying principles which seem to lie close to the experimental findings.

In writing a monograph of this sort the author is in an unenviable position. The field is new, being scarcely ten years old, and consequently information is not as complete as one would desire. Hypotheses to explain the few facts have flourished, and many of them are championed vigorously by their originators.

The author of this book has attempted to summarize most of the facts in this field with which he is familiar, and to place these prominently in front. Generalizations intended to help in the assimilation of these facts have been proposed, and opinions about the applications of the knowledge now at our disposal to practical and theoretical problems have been discussed. It is hoped that most of these opinions will meet the searching criticism of new factual information collected in the future, but it is realized that modifications will be needed in many of them. However, they do not represent casual judgments. Rather they have been weighed carefully over a period of years and are presented as the nearest approximation to the truth which present knowledge has permitted. Many of them were set forth in rudimentary form several years ago in short papers in *Physiological Reviews*, 27, 308 and in *Science*, 100, 579.

Although the present work has been completed (except for a few footnotes) for the past two years, the question of publication of it was much debated in the mind of the author. It had been written with the intention of presenting an intelligible account of experimental results which have been accumulating over the past decade from the efforts of many individuals, and which have seemed unintelligible to some, unconnected to many others, and meaningful to a few. The book deals with the questions and answers from natural occurrences and from human needs which have unfolded to the author from his own experiments and from the reading about those of others.

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June, 1950

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INTRODUCTION

Discovery and development of ideas about antimetabolites

To write history respectably that is to abbreviate dispatches and make extracts from speeches, to intersperse in due proportions epithets of praise and abhorrence . . . all this is very easy, but to be a really great historian is perhaps the rarest of intellectual distinctions. Many scientific works are in their kind absolutely perfect. There are poems which we should be inclined to designate as faultless or as disfigured only by blemishes which pass unnoticed in the general blaze of excellence. . . . But we are acquainted with no history which approaches to our notion of what a history ought to be, with no history which does not widely depart either on the right hand or on the left from the exact line. The cause may easily be assigned. This province of literature is a debatable land which lies on the confines of two distinct territories. It is under the jurisdiction of two hostile powers, and like other districts similarly situated, it is ill defined, ill cultivated and ill regulated. Instead of being equally shared between its two rulers, the reason and the imagination, it falls alternately under the sole and absolute domain of each; it is sometimes fiction, it is sometimes theory.

The perfect historian is he in whose work the character and spirit of an age is exhibited in miniature. He relates no fact, he attributes no expression to his characters which is not authenticated by sufficient testimony, but by judicious selection, rejection and arrangement he gives to truth those attractions which have been usurped by fiction. In his narrative a new subordination is observed. Some transactions are prominent, others retired, but the

scale on which he represents them is increased or diminished, not according to the dignity of the persons concerned in them, but according to the degree in which they elucidate the condition of society and the nature of man.

—LORD MACAULAY, *Essay on History*, London, 1828

The present concept of the antimetabolites has grown slowly with the acquisition of new knowledge of branches of biochemistry. Its roots reach back clearly to the time of Ehrlich, whose Harben Lectures of 1907 (273) present some ideas akin to those which more exact and detailed information has now allowed formulation in more precise terms. The basic concepts have arisen from studies of bacterial nutrition, of animal nutrition, of enzyme action and composition, and of pharmacological antagonisms of drugs. These, and related fields to a lesser degree, have shaped the concept as it now is seen. Along the path of its growth, the work of particular investigators has determined the direction of the search and has provided the framework upon which subsequent experimental observations have been fitted together permanently or until a better structure could be erected to contain them. Some of these outstanding turns in thought will be traced in this introduction in an attempt to see more distinctly how the present position has evolved. In doing so, the judgment of the chronicler may be in error as to details, but the major features should be revealed.

With the investigations of the antimetabolites as with other branches of science, the sequence of events in the emergence of each new idea has usually been first the chance discovery of isolated and unexpected facts. This is then followed by the recognition of the underlying principle. Such recognition frequently results from the bringing together in the mind of one individual of a group of heterogeneous and isolated cases. Each member of this group is seen to be an example of an underlying new phenomenon. The individuals who make such correlations and thus finish the discovery of the new phenomenon are able to do so usually because they carry over a new idea which has been forming in one branch of science and apply it to these heterogeneous chance findings in an allied field. The two stages of discovery are both essential. So long as unpremeditated and unexplained findings are recorded and left to be forgotten they do not constitute discovery. Only when they are activated by an inspiration of the new phenomenon do they become usable and effective knowledge. Finally, as a result of the discovery, a number of explorations are made and from these the validity of the original principle is established. In addition, from these explorations,

the general features of the phenomenon are recognized and classified. Occasionally, these explorations lead to new discoveries which then follow a course similar to that just outlined.

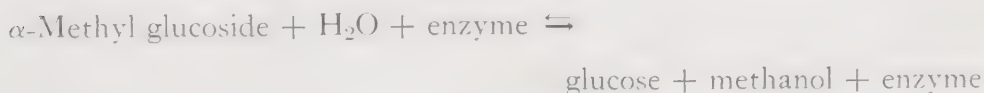
As just indicated, the carrying over of an embryonic idea from one branch of science to another may be followed by a flowering of the transplanted thought in the new field. After it has gained stature and support on the foreign soil, it is frequently brought back again to enrich the land of its origin. These processes can be discerned in an historical account of the antimetabolites.

The inhibition of enzyme action by substances related in structure to the substrate or to the product

Early in the studies of the nature of enzyme action and in the years from 1910 to 1914, Michaelis and his collaborators (230, 274) investigated the inhibition of carbohydrate-hydrolyzing enzymes and observed that some of the inhibitors were similar in chemical structure to the products formed in the reaction. Thus invertase was retarded in its action by fructose, and α -glucosidase was inhibited by glucose. Both these enzymes form the respective inhibitors during the hydrolysis of their normal substrates. This is readily seen by recalling that yeast invertase acts upon sucrose and catalyzes its hydrolysis according to the following equation:



α -Glucosidase may be represented as acting on a substrate such as α -methyl glucoside as follows:



Some residual linking of the product to the enzyme, with the resultant hindering of the action of the latter, was pictured as an explanation of why fructose and glucose were able to cause the inhibition. Such facts and ideas figured prominently in the formulation of the concept about the nature of enzyme action during the early twentieth century.

These studies were limited to inhibition caused by a product of the reaction, and, although these products might be said to be analogs of the substrate, since they do bear structural resemblance to it, the ideas about the matter were clarified when Quastel and Wooldridge in 1927 (114, 275) were able to show marked, competitive inhibition of succinic dehydrogenase by malonic acid, a structural analog of the *substrate* of

the enzyme. They pictured the mechanism of this inhibition much as it is done today, with the possible exception that now some details might be added about the nature of the union of inhibitor with the enzyme. In many of the instances in which the product acted as inhibitor, the structural relationship of the inhibitor to the substrate was clear. The idea that this structural resemblance was the reason for the inhibition gained acceptance in the early part of the twentieth century. By the 1930's, the currently accepted hypothesis to explain competition between substrate and inhibitors related to it in structure was well established among enzymologists. It had not, however, spread to related sections of biochemistry or of biology. The way in which this transition was made is intimately linked with the antimetabolite idea.

The discovery of haptens in immunology

In the years 1920 to 1940, it was discovered that relatively simple substances related in structure to antigens would inhibit the formation of a precipitate when those antigens were mixed with their antibodies. This phenomenon was studied in detail and has been well described by Landsteiner (392) who was largely responsible for its elucidation. The inhibitory substances were called haptens. They were shown to form unions with the specific antibodies, and from these combinations they frequently could not be dislodged by dialysis. One of the major features of a hapten was the occurrence in it of a chemical structure which was also a part of the antigen with which it interfered. In retrospect, it can be recognized how closely the haptens fit into the concept of the antimetabolites, but the realization of this came only after the investigations with antivitamin and antagonistic analogs of hormones, purines, and amino acids had been in progress for several years. Even today the idea is not generally accepted.

Unpremeditated and unexplained findings on the antagonism between certain vitamins, hormones, and amino acids and compounds related to them in chemical structure

In the biochemical and nutritional literature from 1920 to 1940, several instances can be found in which, as a result of chance observations, structural analogs of a vitamin, or a hormone, or an amino acid were discovered to antagonize the biological action of the related metabolite. Such findings seemed remarkable to those who first observed them. In fact, they seemed so unorthodox that critics frequently branded them as errors. A few of these unpremeditated and unexplained observations are noteworthy for the present discussion.

One of the first of such findings, made in 1938 (92), was that β -acetylpyridine and pyridine-3-sulfonic acid were toxic to dogs suffering from nicotinic acid deficiency but were harmless to animals receiving a normal diet. The observations were made during an investigation of various derivatives of nicotinic acid as substitutes for this vitamin, and the possession of toxic rather than beneficial (i.e., vitamin-like) properties in these two analogs was unexpected. After the recognition of the phenomenon of antimetabolites, both of these substances were studied again in a variety of living things and shown to be analogs antagonistic to nicotinic acid.

In 1938, Dyer (84) was interested in the ethyl analog of methionine, viz., ethionine, as a substitute for this amino acid in the diet of rats. She was surprised to learn that when it was given in place of methionine, it caused toxic effects rather than beneficial ones. The astonishment engendered by such an observation is clearly evident in her publication. This toxicity was partially neutralized when methionine was present along with the analog. Ethionine has since proved to be an effective antimetabolite of methionine in a variety of biological systems.

In a series of investigations culminating in 1940, Kuhn (61) and his collaborators ascribed the activity of hormones which elicited male and female behavior in certain algae, not to single compounds, but rather to the ratio between two closely related substances. Either male or female behavior could be called forth by alteration in the proportion of *cis*- and *trans*-dimethylcroctin to which the cells were exposed. At that time the idea that specific biological responses were due to the action of single substances was so universally held, that the proposal ascribing one of these effects to a ratio between two analogs seemed hard to believe. This proposal about the control of specific biological effects still has much opposition but is beginning to gain favor.

Another one of the unpremeditated findings of antagonism between structurally similar compounds was the competition between acetylcholine and allylisopropylcholine (276) and other quaternary ammonium bases (277, 278); and between acetylcholine and atropine (279) which appeared between 1932 and 1937. Observations of these antagonisms were made by pharmacologists who were considerably baffled in attempts to understand them. In fact, the perplexing shift from one type of pharmacological effect to its exact opposite which may result from relatively slight changes in the structure of a drug poses a problem which is not completely explained today, even though the ideas arising from recent studies with antimetabolites may constitute a rational approach to the problem.

Clark's appraisal of the antagonism between acetylcholine and structurally similar drugs

In 1937 Clark published his explanation of the competitive pharmacological antagonism which exists between acetylcholine and tetraalkyl ammonium salts, and between this same hormone and atropine (279). Such antagonisms were then demonstrated by application of the substances to hearts of frogs and observation of the response in contraction. Clark recognized the structural relationship of the drugs to the hormone and saw in this relationship the possible understanding of the mode of action of the drugs. In 1950 his explanation could be improved only by the addition of the analogies of other antimetabolite demonstrations.

He was able from a consideration of these competitive antagonisms between structurally related drugs such as atropine and acetylcholine to conclude that each member of a pair of antagonistic agents probably reacted in a reversible fashion with one special site in the cell. Being a pharmacologist rather than a biochemist, he did not emphasize that one of the antagonists was a metabolite but rather tended to view the hormone as a drug.¹ He recognized, however, that it was of ubiquitous occurrence in animal species and concluded, therefore, that all animals probably had receptor sites capable of combining with it. His deductions about the mode of action of certain drugs represent one phase of the discovery of antimetabolites in relation to pharmacology.² If he had emphasized the metabolite—rather than the drug—properties of acetylcholine, he probably would have been able to make the postulations which recently have energized explorations in chemotherapy.

Clark's conclusions did not find ready understanding or acceptance among pharmacologists, possibly because they were delayed in circulation by the World War, but, within a few years, the field of antimetabolites expanded so rapidly that his contribution was soon merged in a large number of similar views. Biochemical explanations of the mode of action of drugs (the antimetabolite postulate is a biochemical explanation) are usually viewed with suspicion and accepted with con-

¹ Acetylcholine was discovered pharmacologically early in the twentieth century as just another synthetic compound with powerful actions on animal tissues. Not until twenty years later was its natural occurrence and role as a hormone elucidated. It is, therefore, not surprising that its original character as a drug continued to obscure its metabolite function in the minds of investigators.

² Clark recognized clearly that all drug actions were probably not based on competition between structurally similar substances. Quite wisely he pointed out that drug antagonisms were exceedingly common and that his views were applicable only to those cases of competition between pairs of analogous compounds.

siderable reserve in other biological fields. There are far too many good reasons, annotated with experience of the past, why this is probably so.

The discovery of *p*-aminobenzoic acid as an antagonist of the sulfonamide drugs, and postulates about chemotherapy of bacterial diseases which arose from it

In 1940 Woods (34) reported that the bacteriostatic action of sulfanilamide and related sulfonamide drugs was reversed completely and competitively by *p*-aminobenzoic acid. The mode of action of the sulfonamide drugs was therefore pictured as the production in the bacterial cell of a deficiency of the essential metabolite, *p*-aminobenzoic acid. Without this metabolite, the organisms were unable to multiply. *p*-Aminobenzoic acid had not previously been recognized as a biologically important compound, but Woods' prediction that it was such was soon verified amply, both by the finding of several species which could not grow without it (138, 280) and by the recognition of it as an integral part of the vitamin folic acid (281). Woods had been trained at Cambridge where the competitive inhibition of enzymes by structural analogs of their substrates had been discovered, and he had drawn upon this knowledge in making his advance. He was led to his important finding by attempts to isolate a material in yeast extract which was known from the work of Stamp (282) to interfere with the bacteriostatic action of sulfanilamide. Investigation of this substance revealed to him that it was an ether-soluble compound which contained both amino and carboxyl groups, and from this information he was able to predict that it was *p*-aminobenzoic acid. The sulfonamide drugs were thus viewed as structural analogs of this metabolite (see Figure 1, Chapter I) and were thus said to owe their bacteriostatic properties to this fact.

Since *p*-aminobenzoic acid had now appeared as a metabolite, and the sulfonamide drugs as structurally similar antagonists of it, Fildes, in 1940 (283), proposed that useful chemotherapeutic agents against infectious diseases might be produced by altering the structure of some other vitamin or metabolite so as to achieve an antagonistic analog. This idea was immediately tried, especially since at that time the success of the sulfonamide drugs and the coming success of penicillin had raised lush hopes of triumph over bacterial diseases. In fact, the demonstration of Woods exerted such a great influence because it offered a logical basis for the understanding of the action of some highly useful and practical chemotherapeutic agents, i.e., the sulfonamide drugs. By carrying over from enzymology to the fields of bacterial nutrition and

of chemotherapy the knowledge about antagonism between structural analogs, a new direction was given to these subjects.

Largely stimulated by the Fildes' suggestion, but also partly out of curiosity to see if antagonism between vitamins and their structural relatives was a general phenomenon, many investigators soon produced a variety of analogs of bacterial growth factors and showed that they did indeed inhibit the multiplication of a variety of microbial species, and that such effects could be overcome by the related metabolites. Among the earliest studies was that of Fildes in 1941 (129) who showed that indoleacrylic acid antagonized the growth-stimulating properties of tryptophane for the typhoid bacillus. Also, in 1940, McIlwain (87), an associate of Woods and of Fildes, found that pyridine-3-sulfonic acid antagonized the action of nicotinic acid in several bacteria. In 1941, Snell (171) showed that the sulfonic acid analog of pantothenic acid, pantoyltaurine, competed with that vitamin in several bacterial species. This was confirmed in 1942 by McIlwain (173) and Kuhn et al. (105). Robbins in 1941 (284) and Woolley and White in 1943 (5) found pyri-thiamine so to antagonize the action of thiamine. Indeed, studies followed in such profusion that between 1942 and 1947 almost the entire list of compounds to be described in Chapter I was accumulated.

The Fildes' hypothesis, however, that useful antibacterial drugs could be formed by suitable alteration of the structure of a given growth factor was not immediately substantiated. Although such analogs would retard multiplication of pathogenic bacteria *in vitro*, they did not seem to succeed *in vivo*. One reason may have been that this postulate lacked an appreciation of the importance of selectivity of action, and a means of predicting what kinds of structural changes should be made to produce useful agents. Furthermore, the idea did not provide a means of discriminating in the choice of metabolite to be selected for alteration but suggested that any essential growth factor might be expected eventually to yield useful agents. It had not been realized that many of the antivitamins would call forth in animals, as well as in bacteria, the signs of deficiency of the vitamins. Even when this had been shown, some investigators still clung to the hope that the deficiency would prove more injurious to the parasite than to the host, or that it would destroy the former in time to allow salvation of the latter by administration of the vitamin. Time and many failures were required to impress the need for understanding of the basis of selectivity of action. Even today the appreciation of this is not common and frequent studies are based on the assumption that, if an analog of some bacterial growth factor can be designed with very high

potency, it will probably be a promising therapeutic agent (466). An early successful treatment of an experimentally produced infection served to demonstrate the shortcomings rather than to validate the original hypothesis. Thus, McIlwain and Hawking in 1943 (174) showed that rats infected with hemolytic streptococci could be saved from death by huge, often repeated doses of pantooyltaurine. At least two reasons can be seen why this laboratory experiment did not yield a useful therapeutic agent: (1) The drug was excreted too rapidly, and (2) the rats contained too many antagonists of pantooyltaurine, i.e., too much pantothenic acid and other such metabolites. Furthermore, if pantooyltaurine had proved to be one of those antimetabolites which calls forth deficiency diseases in animals, it probably would not have been possible to make even the limited demonstration which was attained.

One success was outstanding among the failures, and this was the development of analogs of pantothenic acid which would in small doses exert a therapeutic effect in experimental malaria. In attempting to learn about the types of structural alteration which would convert metabolites into antagonistic agents, it was found (169) that phenyl-pantothenone, the phenyl ketone analog of pantothenic acid, was quite active in suppressing the growth of many types of microorganisms, but that it was innocuous in animals. The compound was therefore tested as an antimalarial drug and found to be quite able, in small doses, to suppress the blood-induced malaria in several species, including man (186). At about the same time the dibromosulfanilide analogs of pantothenic acid were found to be effective in such infections (185). Part of the success here depended on the chance finding of a selective agent without foreknowledge of how such an agent was to be produced. These pantothenic acid analogs did not elicit signs of vitamin deficiency in higher animals but did so only in microbial species.

The production in animals of signs of vitamin-deficiency diseases with analogs of the vitamins

In a preceding section the chance discoveries were noted that β -acetylpyridine would cause signs of nicotinic acid deficiency in dogs and that ethionine was harmful to rats. One other unpremeditated finding is of interest before the ideas about chemotherapy which arose from the animal experiments are discussed. This example is 3,3'-methylenebis-(4-hydroxycoumarin). The eating of spoiled sweet-clover hay produces a disease in farm animals which is characterized by a marked tendency to bleed profusely from even the slightest injuries, or from places on

the body where friction normally is experienced. This hemorrhagic disease is caused by a specific poison which arises in the hay when it spoils. The agent was isolated by Link and his collaborators (202) and shown to be 3,3'-methylenebis-(4-hydroxycoumarin). The same group of investigators studied the nature of the bleeding which this compound elicited and showed that it was associated with the failure of the blood to clot because of a low prothrombin level. Since this manifestation is the characteristic one of avitaminosis-K, this vitamin was tested for ability to prevent the toxic manifestations of the coumarin and was found to have this property. By that time, the field of antimetabolites was being recognized so that the structural resemblance of the poison to the vitamin became appreciated (compare Figure 4, Chapter 1). However, the antagonism between the two compounds was not of the competitive kind, and, furthermore, very large doses of the vitamin were needed before the action of the coumarin could be overcome. This was not, therefore, a good case for the demonstration without equivocation of the antimetabolite phenomenon.

The demonstration in 1943 (2), that pyriethamine would call forth in mice the typical signs of thiamine deficiency, and that thiamine would competitively antagonize the effects of even many hundred lethal doses of the analog, was a turning point in the discovery of the anti-vitamins. By carrying over the ideas which were forming at that time as a result of the discovery of *p*-aminobenzoic acid in bacterial nutrition to the realm of animal nutrition, the ideas about mechanism of action of structural analogs and of their application to chemotherapy were molded, as will be described presently.

A number of explorations in several laboratories rapidly revealed that properly constituted analogs of all the other water-soluble vitamins, and of some of the fat-soluble ones, would call forth in animals the signs of deficiencies of the related metabolites. Thus, in 1943 (26) glucoascorbic acid was found to elicit a condition in mice similar to that seen in scurvy of man, and in 1944 the phenazine analog of riboflavin (109) as well as isoriboflavin (107) were observed to produce ariboflavinosis in mice and rats. Mentzer and Meunier (206) who recognized the probable antivitamin properties of 3,3'-methylenebis-(4-hydroxycoumarin) synthesized some other relatives of this vitamin and showed that they lowered the prothrombin level of the blood of rabbits. Ott (101) found in 1946 that administration of desoxypyridoxine to chickens resulted in the appearance of a condition resembling that which accompanies a lack of pyridoxine. Likewise, α -tocopherol quinone was observed to cause the development of signs of vitamin E

deficiency in pregnant female mice (127). After such demonstrations had been made with the vitamins it was only to be expected that analogs of some of the hormones and other essential metabolites would be made and shown to call forth the characteristic signs of deficiency, and this actually occurred. However, such antimetabolites have not appeared in such numbers as have those derived from the vitamins.

The demonstrations with animals were important from a theoretical standpoint for two reasons. Firstly, they provided the best evidence that antimetabolites actually functioned by creating in some way a deficiency of the related metabolite. The experiments with sulfanilamide and with pantooyltaurine and with pyridine sulfonic acid were done with bacteria, and they showed only that a competitive antagonism in the promotion of growth existed between metabolite and analog. It was by no means certain that these agents did actually produce deficiencies of their respective vitamins in the bacteria although they were postulated to do so. These were the chief examples known at the time that the work on animals was done with pyriethamine. When a normal animal, by administration of small doses of pyriethamine, could be made to exhibit all the manifestations characteristically associated with thiamine deficiency of dietary origin, it became much clearer that the induction of a metabolic lack of the vitamin was the probable mode of action. When a competitive type of antagonism could then be shown between vitamin and analog, as could be done with thiamine and pyriethamine, the argument was stronger. Finally, when the effects could be understood in terms of the inhibition of enzymes by structural analogs of their substrates, a clear hypothesis about mechanism of action could be formulated.

The second idea of theoretical, and probably of practical, importance which arose from the experiments with antimetabolites in animals was that dealing with the formulation of a new means of discovering pharmacological agents, which will now be discussed.

Emergence of hypotheses about the designing of chemotherapeutic and other pharmacological agents for non-infectious diseases

Because Woods' discovery with the sulfonamide drugs dealt with the chemotherapy of infectious diseases, and also because of the intense interest in treating infections which these drugs and penicillin had engendered, the Fildes' hypothesis about the way to proceed dealt with agents which might be active against infections. Indeed, the whole subject of antimetabolites acquired a flavor of bacteriostasis in its infancy which threatened to stunt it. The chemotherapy of infectious

diseases is only one part of the field of chemotherapy, and the ideas of antimetabolites began to develop in several laboratories in relation to the other parts as well.

A paper in 1944 (205) recognized that new types of interesting and possibly useful pharmacological agents were being realized with the discovery of the antivitamin. The ideas were illustrated in the following way. 3,3'-Methylenebis-(4-hydroxycoumarin) had been found to be the first member of a group of drugs which would lower the prothrombin level of the blood (202). This characteristic effect of vitamin K deficiency had a clinical usefulness, and agents which would elicit it were previously not known. Similarly, for pyriethamine, a first member of a new series was at hand which would call forth in animals those pharmacological manifestations which are seen in a dietary lack of thiamine. If these had usefulness either in the clinic or in the pharmacological laboratory, a means was available for their production. From the extensive knowledge of the effects of each of the several deficiency states it was considered probable that drugs which would call forth various predictable manifestations could be foretold, and then realized. Furthermore, it was recognized that there were general ways in which the structure of a metabolite could be altered in order to produce an antimetabolite, and that each type of alteration did not yield compounds of the same qualitative action. Within the next three years, these ideas were given support by several laboratory experimental models, as well as, in one instance, by limited clinical success.

The ramifications of this idea in relation to practical and theoretical pharmacology will be discussed in a succeeding chapter, but, for the historical perspective, it may be of interest to mention two of the early examples arising from it. Since thyrotoxicosis is believed to result from an excess of the hormone thyroxine in the body, an analog which would nullify the excess might be of benefit. It has not been determined clinically whether such an analog is of value, but it was quite possible to predict and to synthesize substances which would antagonize the action of excess administered thyroxine in experimental animals (128). The analogs of folic acid such as 4-aminopteroylglutamic acid, which are said to give some temporary remissions of leucemia in children (285) have been developed on this basis, since it is well known that a lack of folic acid results in marked reduction in the white cells in the blood, and it is this reduction which is desired in leucemia. No drug of lasting usefulness³ has yet been developed as a result of ap-

³ This statement may seem erroneous to some investigators who would point out to us that the antihistamine drugs have found lasting usefulness in medicine. However, these agents were not originally developed by *conscious* alteration of the struc-

plication of this hypothesis, but it is possible that one may be. It is also quite possible that, as with the Fildes' hypothesis for prediction of antibacterial agents, much elaboration to encompass the problems of selectivity of action and of localization of compounds in specific tissues will have to be solved.

Clark had left the explanation of the mode of action of certain drugs at the stage where competition for a specific site of action was envisioned. Thus, for structural relatives such as atropine and acetylcholine the competitive biological antagonism was taken as strong evidence that both substances acted at a single site. The concept was somewhat befogged by the view of a metabolite such as acetylcholine as just another drug. The contribution which the experiments with antivitamin made to the understanding of drug action was the clear picture of one of the competing agents as an essential metabolite, not harmful to the cell but rather required for its normal function. This view was possible with the vitamins more readily than with the hormones because, with vitamins, no effect on the normal animal could be seen even with large doses. A deficient individual was needed before their importance could be shown. With the hormones, on the other hand, doses slightly in excess of those normally present in the tissues brought about profound pharmacological effects. It was thus quite natural to regard them as drugs and hence to becloud the importance of the opposing actions with the structural analog. With the antivitamin the analog by itself showed a profound effect on the animal, but the metabolite was innocuous when tested alone in a normal individual. This situation was more conducive to clear understanding than was that involving hormones.

Development of ideas about selectivity of action and how to achieve it

For a long time it has been recognized that some drugs affect only certain organisms, or only certain parts of an individual, without causation of histamine. Rather, they arose from classical pharmacological experiments designed to note the effect of changes in the constitution of a drug on its biological activity. Subsequent to the recognition of the antimetabolite phenomenon, the knowledge gained in its study was in some degree used in perfecting antihistamines, but even then the most useful agents were not first developed as structural analogs of histamine. In fact, there was considerable reluctance towards accepting antihistamines as antimetabolites. It is frequently easy to look backwards and to suppose that the explanations of how various phenomena are caused should have been obvious long before they were. Indeed, the most vigorous critics who challenge the explanation when it is first offered subsequently may not be able to realize that there should have been any opposition to the idea. Once an hypothesis gains favor, it is often used without discrimination to explain all sorts of natural occurrences.

ing any alteration in other parts. This selectivity of action is one of the prime requisites of a useful therapeutic agent. Although this conviction was prominent in the early experiments of Ehrlich with arsenical compounds, knowledge was insufficient at the time to allow much progress beyond the finding that susceptible organisms bound the drugs. In an earlier section of this introduction it was seen how the hypotheses arising from the discovery of *p*-aminobenzoic acid fell short of yielding useful drugs immediately, partly because of a lack of understanding of this problem. Practically the only success in the use of antimetabolites for the treatment of infectious diseases, namely, that of pantothenic acid analogs in malaria, was based on the chance observation that the analog was harmless to animals, but still toxic to microorganisms. In fact, the problem of selective toxicity was left largely to chance. The aim of most studies was to find a poisonous substance and to hope that it would not harm the host unduly. The realization of the paramount importance of selective action arose slowly from a series of failures which accompanied reliance on chance, but this realization gradually stimulated thought and experimentation to learn of the underlying mechanism, so that selective agents might be predicted. These endeavors are still in their formative stages and are proceeding in the directions to be discussed in Chapter 6. However, some of the early observations which are influencing the thinking may be of interest in gaining an historical perspective. One of the first was that many antimetabolites retard those organisms which have a nutritional requirement for the related metabolite, but do not harm those which do not. This unpremeditated finding was first made with pyridine-3-sulfonic acid by McIlwain (87), with pantoyltaurine by Snell (171), and with pyriethamine (5). Another early idea indicated that selectivity depended on differences in the metabolic machinery of various species (450). This arose partly from the observation that bacteria which lacked the ability to synthesize folic acid were not affected by sulfanilamide and its derivatives. Since these antimetabolites inhibit the synthesis of folic acid, it became understandable why organisms which could not carry out the synthesis were not affected. This deduction of Lampen and Jones (245) was soon applied to the explanation of the resistance of higher animals to these drugs (286) because these species do not form folic acid. Explorations with other metabolic systems lent support to this idea (314). Such studies and ideas have not yet provided the insight necessary to yield useful drugs, but it is clear that investigators are now passing from a recognition of the problem into planned experimentation in an effort to make the next turn in thought.

Relationship of antimetabolites to the etiology of some diseases and to normal physiological processes

After a number of synthetic antimetabolites had been studied, it became increasingly clear that such compounds also existed in nature and that they were sometimes etiological agents in the production of disease, or were concerned in normal physiological processes. Some of the oldest examples of this were not recognized to represent antimetabolites at the time of their discovery, and only as the work with synthetic structural analogs progressed did the older findings receive new interpretation. For example, the fact that 3,3'-methylenebis-(4-hydroxycoumarin) caused signs of vitamin K deficiency, and even the finding of the reversal of this action with the vitamin, came some time before any attempt was made to relate this naturally occurring causative agent of the hemorrhagic sweet-clover disease to the antimetabolites. The structure and role of this substance in the disease were understood in 1941 by Link and his collaborators (287), but a few years elapsed before the possible relationship of the etiologic agent to the antimetabolites was recognized (205, 206).⁴

A second case, namely, that of the pellagragenic agent of corn (288), shows the direction in which thought had gone. By 1946, the possibility of the existence of naturally occurring antimetabolites and of their role in the etiology of certain diseases prompted a search for a toxic agent in corn. Such a poisonous substance could be extracted from the grain and could be shown to antagonize the action of nicotinic acid in mice and rats. Although the active principle has not been isolated in pure form, its chemical behavior suggested that it might be a pyridine derivative and possibly an analog of the vitamin. If this should prove to be so, then this antinicotinic acid might play a role in the etiology of pellagra among corn eaters.⁵

Contemporary with the recognition of the possible parts played by antimetabolites in the etiology of certain diseases, their functioning in the normal physiological control of metabolic processes began to be considered. The antagonism between pairs of structurally similar metabolites in the regulation of normal physiological processes was

⁴ Another naturally occurring structural relative of vitamin K was the first to be regarded as a naturally occurring antimetabolite. This was iodinin, an antibiotic pigment of *Chromobacterium iodinum*. McIlwain in 1943 (222) recognized that this compound, a dihydroxyphenazine oxide, was related in structure to vitamin K and that this metabolite would counteract its effect on the growth of bacteria.

⁵ As might be expected, this is not the only factor in the etiology of pellagra because the amounts of nicotinic acid and of tryptophane and possibly of other substances in the diet probably are important.

first found accidentally, without premeditation. Thus, Kuhn and his collaborators observed the control of sexual activity in algae by varying mixtures of *cis*- and *trans*-dimethylcroctin. This was in 1940. By 1946 (118, 119) the well-known antagonism which exists between androgens and estrogens in higher animals was beginning to be regarded as a similar regulation of sexual character in these species. The marked structural similarity of testosterone and estrone had been known for several years. Likewise, it had been found that, surprisingly enough, estrogens were excreted by males and androgens by females. This reversion of the occurrence in an individual of the hormones which his sex should demand was perplexing to endocrinologists, but it could be understood if both kinds of hormones participated, by virtue of their antimetabolite action towards each other, in the determination of the final expression of sex. The balance of the two would be tipped one way in males and the other in females. Such an idea gained ground slowly but considerable evidence can be cited in its support. (See Chapter 5.)

Among the amino acids, a similar idea developed to explain the antagonisms which can be demonstrated between pairs of these metabolites. Quite unexpectedly, Gladstone in 1939 (289) observed that the growth of *Bacillus anthracis* was inhibited by valine and that this effect was overcome by leucine. Several other pairs of structurally similar amino acids were found to be mutually antagonistic. No adequate explanation of such behavior could be offered in 1939, but a few years later two associates of Gladstone, namely, Woods and Fildes, might have been able to do so. Another unpremeditated finding was that of Doermann in 1944 (82). He observed an antagonism between lysine and arginine in the growth of a lysine-requiring mutant of *Neurospora*. Such antagonisms of structurally related pairs of amino acids were soon studied in considerable profusion, because investigators began to recognize that they might be examples of naturally occurring antimetabolites and that the delicate control of physiological processes might be accomplished in part by the utilization in the cell of this phenomenon. Such an idea is still a postulate but the antagonism is real.

As more and more cases of this sort came to light as the result of casual or of planned explorations, it became increasingly clear that the phenomenon of antagonism between structurally similar compounds was probably being used in nature to a considerable extent. The detailed discussion of the evidence for this point of view has been presented in a separate chapter on this aspect of the problem. The emergence of the idea has seemed to follow the course just outlined.

Repercussions in enzymology

Having started out from the field of enzymology and penetrated into nutrition and into pharmacology, the idea of antagonism between a substrate and its structural analog has again been brought to bear in problems of enzymology. While the idea was abroad, much was learned of how to make analogs which would be good antagonists, and even the basic concept of the mode of action improved markedly. By use of the accretions thus collected, a better understanding has developed of some phases of the mode of enzyme action. Retardation by products of an hydrolytic enzyme reaction gave some clue to the structures involved in the enzyme-substrate complex, as the early experiments of Michaelis had indicated. More recent investigations with analogs of the substrate have added to this information. For example, Neurath and his collaborators (290) have been able to form specific inhibitors for certain proteolytic enzymes, and from a study of these they have begun to define something of the nature of the enzymatically active centers of such proteins.

Such applications of antimetabolites to the study of the nature of enzyme action have not been pursued so vigorously as has another branch of enzymology, namely, the use of these agents to discover or to explore a chain of reactions by means of which metabolites are synthesized or transformed in living organisms. One of the first of such uses resulted in the recognition of the probable pathway of purine synthesis. Subsequent studies of other processes were influenced markedly by the results of this one.

Various pieces of this investigation have been discussed in appropriate, succeeding chapters of this book, but a chronological recounting of some of the salient findings may, without redundancy, show the development of the idea. Harris and Kohn (85) observed that the bacteriostatic action of sulfanilamide was overcome, not only by *p*-aminobenzoic acid, but also by methionine. They pointed out that this could be understood if methionine were a product of some metabolic reaction system in which *p*-aminobenzoic acid participated, perhaps as a coenzyme. Some of the purines were then found likewise to antagonize the action of sulfanilamide, and a similar explanation was advanced for them as well. This idea was elaborated by Shive and Roberts (235) under the name of inhibition analysis. The character of the antagonism was taken to indicate the relationship to each other of substances which were able to influence the action of an antimetabolite (253), and to conclude about the sequence of the reactions involved. Although some of the conclusions have not persisted in the face of

further study of the reactions, the general idea that structurally dissimilar, non-competitive antagonists may be products of a series of reactions in one of which the structurally similar metabolite is participating has frequently been corroborated.

While these developments were in progress, apparently unrelated studies began to impinge upon them. The idea that the most probable point of action of antimetabolites such as sulfanilamide was the competition with the structurally similar metabolite (such as *p*-aminobenzoic acid) acting as a substrate, rather than as a coenzyme, was being recognized (118, 248). This idea received support from the work arising from the elucidation of the structure of folic acid (281). Because *p*-aminobenzoic acid was found to be an integral part of this vitamin, the latter could readily be conceived as a product of a series of reactions in one of which the former was substrate. Nimmo-Smith et al. (141) soon demonstrated by classical enzymological studies that this was probably so and that sulfanilamide did inhibit the formation of this product.

At about this time, Stetten and Fox (367) isolated an amine, $C_4H_6ON_4$, from bacterial cultures grown in the presence of sulfadiazine. Reflecting on the postulate of inhibition analysis, and recalling that purines antagonized the action of sulfonamides, Shive et al. (325) recognized that this amine could be 4-amino-5-carboxamidoimidazole, because the addition of one carbon atom to it would yield a purine (hypoxanthine). They showed that the amine was, in fact, this imidazole. Presumably it was a metabolic precursor of the purines which accumulated when the normal synthetic reactions leading to these compounds were blocked by the sulfonamide analog of *p*-aminobenzoic acid. This idea has received support from some subsequent studies in which whole animals, or liver homogenates, were observed to incorporate radioactive 4-amino-5-carboxamidoimidazole into hypoxanthine (459, 476) and other purines. Nevertheless, there are some objections to the acceptance of this imidazole as a purine precursor.

Although the investigations just outlined had implicated *p*-aminobenzoic acid in the formation of purines, it became increasingly clear that this substance was only secondarily concerned, and that folic acid was more nearly related to the process. A nutritional study had revealed (477) that adenine plus thymine could partially replace folic acid for the growth of *Lactobacillus casei*. Presumably this was because folic acid was functioning in the formation of these compounds. In addition several experiments with antimetabolites of purines, pyrimidines, and pterins (see Chapter 9) were recorded which likewise indicated some metabolic interrelationships of folic acid and purines. This interrelationship was seen more clearly when it was found that aminopterin,

an antimetabolite of folic acid, brought about the accumulation of 4-amino-5-carboxamidoimidazole in cultures of bacteria just as sulfadiazine did (370). This seemed to indicate that the effect of the latter was merely to cause a deficiency of folic acid, and that it was the lack of this metabolite which allowed the imidazole to appear. Extended studies with other antimetabolites as well as some nutritional investigations have given rise to the further belief that the formation of a desoxyriboside of the imidazole or of a purine is an intermediate step in this chain of reactions concerned with folic acid, but the evidence for this has not been clarified sufficiently to justify a discussion of it here.

These studies with antimetabolites have suggested that the purines are formed by a series of reactions in which *p*-aminobenzoic acid is incorporated into folic acid, which then functions in a number of processes which lead to the purines, probably by the insertion of a carbon atom into 4-amino-5-carboxamidoimidazole, or some derivative of it. The outline of this biosynthetic reaction chain is fragmentary, since only a few points have been glimpsed. Like travelers on a winding mountain road seen from afar, their number and sequential relationship are not entirely clear. The facts which have been found, however, have served as points of orientation from which more detailed explorations by classical techniques of enzymology are being initiated. The origination and use of these ideas arising from the antimetabolites have been a new development in enzymology.

The exploration of the principles of the phenomenon of antimetabolites

The general principles underlying the phenomenon of antimetabolites, in so far as they are known at present, have been explored by many investigators in the field. This has been the descriptive phase of the discovery, and from the minute explorations of many laboratories have come the principles as they are known today. Generalizations when they are enunciated must rely on a body of factual information accumulated from the experiences of many. The recognition and statement of the generalization sometimes prove to be a considerable advance in knowledge.

CHAPTER 1

Competition between metabolically important compounds and substances related to them in chemical structure

Introductory remarks

As was indicated in the introduction to this book, the past decade has witnessed the intensive study of biological antagonism between structurally similar substances. In most instances one of the pair of antagonists has been a chemical compound which is essential to the existence or the activity of living things. Such substances, because they do play decisive roles in metabolic reactions, have frequently been called "essential metabolites" or more simply "metabolites." They may be vitamins, or hormones, or amino acids or other compounds which occur in nature and which can be shown to be necessary in some phase of the life process.

The structural analogs of these metabolites are frequently synthetic compounds produced in the laboratory, but occasionally they may also be found to occur naturally. They are of interest in this book because they may show the property of calling forth in living things the signs associated with a deficiency of the metabolite to which they bear structural resemblance. In almost all instances, this biological effect may be overcome merely by increasing the concentration of the metabolite. These antagonistic structural analogs have been called antimetabolites. When the metabolite in question is a vitamin the analogs have been called antivitamins, and when it has been some other substance such as a purine they have been termed antipurines, etc. Although the more precise name would be "structural analogs antagonistic to metabolites," the loose generic name antimetabolites has been more generally used.

The biological effects of the antimetabolites may be seen in a variety of ways. It may be the production of characteristic signs of a vitamin

deficiency disease in higher animals. It may be the inhibition of the growth of bacteria. It may be the demonstration of a chemotherapeutic effect on an infectious disease of man or other animals, or it may be the eliciting of a beneficial response in non-infectious diseases. The effects may not be visible at all in intact organisms and may be seen only as an inhibition of some isolated enzyme system. Frequently, however, the effects on living things may be understood in terms of specific interference with isolated metabolic reactions *in vitro*.

Because of the rapid increase in experimentation with antimetabolites it has seemed desirable to assemble some of the salient facts which have been established and to attempt to conclude from these something about the nature of the basic phenomenon and to draw from this some suggestions about how best to proceed. Therefore, it is proposed in this chapter to discuss a few experiments in some detail so that the basic phenomenon can be appreciated. After this, it is intended to mention briefly most of the other instances in which an antimetabolite demonstration has been made with a structural analog of some metabolite. The purpose is chiefly to record established facts in this chapter and to leave to subsequent sections a discussion of the correlations and uses and opinions which have arisen from these facts. No attempt has been made to write a chronological account in which each fact would be placed in sequence with those which came before and after. For this reason the metabolites have been divided into classes and within each an alphabetical sequence has been followed. The aim has been not to establish historical precedent but rather to describe the basic phenomenon by use of examples which had some historical importance (not necessarily the first instance), and then to record as concisely as possible similar observations with other metabolites.

The basic phenomenon

If the chemical structure of a given metabolite is changed in one of several minor ways, a compound may be obtained which will call forth signs associated with the specific lack of the metabolite when the analog is given to animals, or microorganisms, or other living things. This is the basic phenomenon with which we shall be concerned in this monograph. The various ramifications of this central theme will be explored in succeeding chapters, and some of them will be seen to lead to results of theoretical and practical importance. For the present, however, let us consider some of the examples of the basic phenomenon so that we shall have a collection of facts with which better to approach an understanding of the mechanism and uses of it.

The structural analogy between the antagonist, or antimetabolite, and the metabolite will be considered to be a basic tenet. Although it is true that many diverse substances, and even changes in physical conditions, may be antagonistic to the action of certain biologically important compounds, the inclusion of such examples in the present discussion would becloud rather than clarify the issue. Thus, starch or sugar can be shown to be antagonistic to the action of thiamine, since the thiamine requirement of an animal may be increased by raising the carbohydrate content of the diet. Likewise, calcium carbonate may be said to antagonize the action of vitamin D, since the addition of large quantities of it to an animal's ration may render insoluble or otherwise bind some of the phosphates contained therein, and thus tend to increase the requirement for vitamin D.

Again, certain substances may react chemically with a biologically essential compound and destroy it. For example, sodium sulfite, when added to a ration, will destroy the thiamine, or rancid fats, when mixed with the food, may oxidize vitamin A. The destruction by these chemically reactive materials may also occur *in vivo*, as when rancid fats reduce the already existing store of vitamin A in an animal's liver. Although both sodium sulfite and rancid fat might be said to be antagonistic to metabolites, such examples of simple chemical destruction of the active substance must be excluded from the present discussion.

Changes in physical environment may sometimes appear antagonistic to specific metabolites. For example, strains of the fungus *Neurospora* are known (1) which require riboflavin when grown above 25°C., but do not need this vitamin in the medium at lower temperatures. Elevated temperature might thus be said to be antagonistic to riboflavin.

The causes of such antagonisms are so diverse, and frequently are so obscure, that to bring them under the heading of antimetabolites would take us into a discussion of all of biochemistry. On the contrary, those biological antagonisms which exist between metabolites and their structural analogs seem to have a single underlying basic cause, and hence a unity which justifies the consideration of them as a class. This class of substances will be called the antimetabolites. A more precise term would be "structural analogs antagonistic to metabolites."

It should be clearly understood that all analogs of a metabolite are not antimetabolites of it. This can be appreciated readily when we consider that an analog is merely a compound which bears structural resemblance to the metabolite. An antimetabolite has not only this structural resemblance, but also biological properties which relate its action on living things to that of the metabolite.

The inhibition of bacterial growth with sulfanilamide, and its prevention with *p*-aminobenzoic acid

Shortly after the discovery that sulfanilamide (*p*-aminobenzene sulfonamide) would inhibit the growth of many kinds of bacteria (250), it was recognized that the harmful effects of this agent could be nullified by a component of the broth in which the organisms were grown. Materials such as extracts of yeast or of streptococci likewise increased the amount of sulfanilamide required to retard growth in media to which these extracts had been added (282). Woods attempted to isolate this antagonistic substance from yeast extract and was able to show that it was an amphoteric compound of high activity in comparison to the amount of sulfanilamide which it antagonized (34). Very small amounts of the partially purified extract nullified the bacteriostatic action of moderately large concentrations of the drug. Because it was known among enzymologists that certain enzymic reactions could be inhibited by addition to the system of a compound related in structure to the normal substrate, Woods suggested that the antagonist to sulfanilamide, which was found in living cells, was probably a structural analog of the drug. He postulated that it was *p*-aminobenzoic acid. The structural resemblance may be seen in Figure 1.

p-Aminobenzoic acid was very active in overcoming the inhibition of growth of bacteria which sulfanilamide caused. Furthermore, the antagonism was competitive. Thus, if 50 gammas of sulfanilamide were needed to stop the growth of a strain of hemolytic streptococci when 0.007 gamma of *p*-aminobenzoic acid was present in the medium, three times as much was required when three times as much *p*-aminobenzoic acid was added. Similarly, ten times as much was required when the concentration of *p*-aminobenzoic acid was raised tenfold. These facts are illustrated by the data in Table 1, which have been taken from the work of Woods (34). Thus, the toxicity of sulfanilamide for these bacteria could not be defined in terms of the concentration of it in the medium, but rather had to be stated in relation to the amount of *p*-aminobenzoic acid which was simultaneously present. Amounts of the former which were quite poisonous became harmless when the concentration of the latter was raised sufficiently. This com-

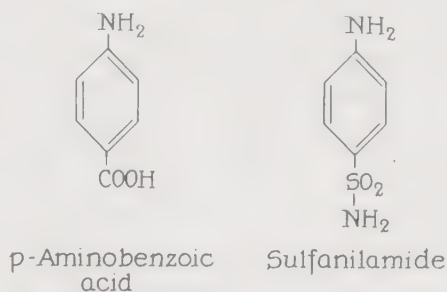


Figure 1. Structures of *p*-aminobenzoic acid and sulfanilamide.

Table 1

Quantities of sulfanilamide required to inhibit the growth of streptococci in the presence of various amounts of *p*-aminobenzoic acid

Concentration of Sulfanilamide	Concentration of PAB Required
$M \times 10^{-3}$	$M \times 10^{-7}$
0.30	0.58
1.52	2.91
7.58	14.54

petitive character of the antagonism was an important feature which has assumed prominence in the interpretation of the phenomenon.

Woods' discovery of the antagonism between sulfanilamide and *p*-aminobenzoic acid led to the belief that *p*-aminobenzoic acid was an essential metabolite which functioned in the growth of bacteria. Evidence in favor of this view accumulated quickly, because *p*-aminobenzoic acid was found to be essential to the growth of several kinds of microorganisms. Some species which did not require it were shown to synthesize it for themselves (139). Furthermore, this substance was found to be an integral part of another vitamin (pteroylglutamic acid or folic acid) (281). Its importance was thus clearly established.

The antagonism between sulfanilamide (or its derivatives) and *p*-aminobenzoic acid may be shown in the biological synthesis of folic acid. This has been done both with growing bacteria and with resting cells. Thus, when *Escherichia coli* is grown in the presence of sub-inhibitory concentrations of the drug (i.e., amounts just insufficient to inhibit growth), the quantity of folic acid found in the culture is much less than when the drug is absent (244). The demonstration with resting cells has been carried out with *Streptobacterium plantarum* (141). Cell suspensions of this organism under suitable conditions form folic acid when *p*-aminobenzoic acid is added as a substrate. This synthetic activity is inhibited by sulfanilamide or its congeners. An enzymic system in the resting cells has been presumed to carry out this synthesis upon which the analogs exert a competitive inhibition. Thus, the antagonism can be demonstrated without growing cells.

Because the sulfonamide drugs have had wide clinical usefulness, intensive study has been made of a large number of such compounds. The great array of derivatives of sulfanilamide which have been tested for antibacterial powers has been the subject of many reviews such as that of Northey (140). All such compounds which have shown therapeutic effects in infectious diseases are related to *p*-aminobenzoic acid in that the carboxyl group has been replaced by some other more or less acidic radical. This is usually a sulfonamide but is not invariably

so. Usually, one of the hydrogen atoms of the sulfonamide is replaced by a ring system such as a thiazole or pyrimidine, in order to render the compound more active than sulfanilamide both *in vitro* and *in vivo* against bacteria. Thus, sulfathiazole is a more potent inhibitor of bacterial growth than is sulfanilamide. Nearly all these congeners of sulfanilamide are antagonized competitively by *p*-aminobenzoic acid in their action on growing bacteria.

Although the sulfonamide drugs cause inhibition of growth of many kinds of microorganisms, and although this effect may be overcome with *p*-aminobenzoic acid, these substances do not cause a condition in animals which can be recognized as a deficiency of this metabolite. The elimination of *p*-aminobenzoic acid from the diet of laboratory animals does not result in the production of a characteristic deficiency disease,¹ or, in other words, this metabolite is not a vitamin for these animals. Therefore, the signs of deficiency of the metabolite are unknown. If it were possible to recognize a characteristic train of manifestations of disease, and then to produce these signs by administration of sulfanilamide, one could say with more certainty that the analog caused a deficiency of the metabolite. Since this has not been done, with the sulfonamides and *p*-aminobenzoic acid the conclusions rest on the inhibition of microbial growth and its reversal by the metabolite, and on the inhibition of the enzymic synthesis of folic acid. The additional evidence of production of characteristic signs of deficiency of the metabolite in animals is lacking.²

Production of thiamine deficiency in mice with pyriethamine (neopyriethamine, heterovitamin B₁)

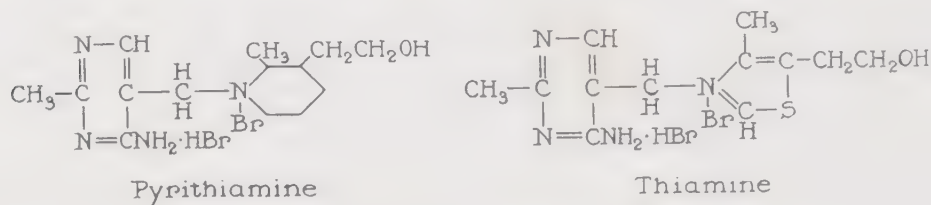
If the sulfur atom of the vitamin thiamine is replaced by a vinyl group, i.e., by $-\text{CH}:\text{CH}-$, an agent is produced which is antagonistic

¹ Some reports in the literature have described a retardation of growth of rats deprived of *p*-aminobenzoic acid. However, many other investigators have failed to observe in animals any vitamin-like properties for this compound, so that the prevailing opinion now seems to be that under ordinary conditions it is not a dietary essential.

■ The fact that the feeding of sulfonamide drugs to rats or mice under specialized conditions results in the production of signs of deficiency of folic acid might be cited as evidence that the drug had caused a sign of deficiency of *p*-aminobenzoic acid in these animals. It might be argued that this is similar to the situation in the experiments with resting bacterial cells where the analog competed with the metabolite with the end result that folic acid synthesis was inhibited. However, the evidence with rats is somewhat indirect, and the issue in animals has been beclouded by claims that the sulfonamides act on microorganisms in the intestines of the animal rather than on the rat itself (143).

to the vitamin. This antimetabolite is the pyridine analog of thiamine and is known as pyriethamine (2).³ The structural relationship is shown in Figure 2. Pyriethamine may be synthesized (3) by a long series of reactions which leads to the formation of 2-methyl-3-hydroxyethylpyridine. This is then coupled with the pyrimidine portion of thiamine (2-methyl-4-amino-5-bromomethylpyrimidine) preferably under the improved conditions recently described (4).

If small doses of pyriethamine are given to mice maintained on an adequate diet of highly purified rations of known thiamine content, signs closely resembling those of thiamine deficiency are produced (2).



slight noise made while the animals are sitting quietly in their cages. After a stage of incoordination of movement of legs in walking, and bouts of violent retraction of the head upwards and backwards (opisthotonus), a condition is reached in which the mouse lies on its belly with the legs extended to the sides at right angles to the body. Death usually follows this stage within a few hours. In the symptomatic interval, and possibly slightly before, profound inanition develops, accompanied by loss of weight.

The train of pharmacological signs elicited by pyriithiamine is quite similar to that seen in various species suffering from acute thiamine deprivation, but in mice these signs are more extensively developed in pyriithiamine-treated animals than in those merely deprived of the vitamin in the diet. Needless to say, the basal ration alone, without addition of the analog, contains sufficient thiamine to maintain the animals in good health and to promote growth.

The vitamin has the ability to prevent the disease, or to cure it. Thus, if the thiamine content of the ration containing pyriithiamine was raised, the analog proved to be innocuous. Furthermore, animals

Table 2

Response of mice to thiamine and pyriithiamine

Amount * of Pyriithiamine, milligrams per day	Number of Animals	Amount of Thiamine, gammas per day	Time to Produce Symptoms, days	Number of Animals Showing Symptoms	Average Weekly Weight Change, grams
0	35	1.6		0	+3.0
5	5	1.6	5	5	-3.0
0.5	12	1.6	7	12	-2.7
0.3	5	1.6	6	5	-2.1
0.15	5	1.6	8	5	-0.2
0.08	5	1.6	10	5	+1.9
0.025	5	1.6	11	5	+2.5
0.025	8	2	12	6	+2.4
0.013	3	2		0	+3.5
0.15	5	61.6		0	+3.1
0.5	8	60		0	+3.6

* Amounts are expressed in terms of pure pyriithiamine. Compare footnote 3 of this chapter. In other respects, the data have been taken from those in the literature (2).

taken even at the terminal stages, in which they are lying with legs outstretched, responded within an hour or two when given an oral dose of thiamine, and recovered completely within a day following this therapy.

The intensity of the deficiency may be measured most objectively by noting the effects on rate of gain of body weight, and on mortality. Representative data of this type obtained with mice fed graded doses of pyrithiamine and various levels of thiamine are shown in Table 2. These data indicate clearly that the toxicity of pyrithiamine depends directly on the amount of thiamine simultaneously ingested. If one were to determine the minimal lethal dose of the drug, one would find that twice as much was required to kill a mouse getting 4 gammas of thiamine per day as for one receiving only 2 gammas, and that ten times as much would be needed for an animal getting 20 gammas of the vitamin.

Mammalian species other than mice have also been shown to exhibit signs of thiamine deficiency when they receive pyrithiamine.

Inhibition of growth of microorganisms with pyrithiamine and its reversal with thiamine

The ability to produce inhibition of growth and other signs of thiamine deficiency can be demonstrated not only in mice and rats but also in a wide variety of living things. When pyrithiamine was added to an appropriate culture medium, the growth of various bacteria and fungi could be inhibited. As with mice, so with these organisms; the deleterious effects of the compound could be erased completely by simultaneous addition of increased amounts of thiamine. Therefore, the inhibition of growth caused by the pyrithiamine was ascribed to the production of a thiamine deficiency in the organisms. Data to show the concentrations of the drug needed to inhibit the growth of such microbial species cultivated in the presence of a constant amount of thiamine are shown in Table 3. Of all the species tested, only those requiring thiamine, or one of its constituent parts, were affected. Those which had no nutritional need for the vitamin (and which presumably made their own supply of it) could withstand large concentrations of the analog. These data are taken from the literature (5).

With the microorganisms a competition of the vitamin and its analog could be demonstrated just as could be done with mice. Thus, the concentration of pyrithiamine needed to cause half-maximal inhibition of growth was twice as great if the thiamine content of the medium was doubled, and ten times as great if it was multiplied by 10.

Table 3

Inhibitory power of pyriethamine for various microbial species grown in the presence of 0.01 gamma of thiamine per cubic centimeter

Organism	Amount * to Cause Half Maximal Inhibition, gamma per cc.	Thiamine Requirement
<i>Ceratostomella fimbriata</i>	0.02	Intact thiamine
<i>Ceratostomella</i> from London plane tree	0.05	Intact thiamine
<i>Ceratostomella pennicillata</i>	0.03	Intact thiamine
<i>Phytophthora cinnamomi</i>	0.03	Intact thiamine
<i>Chaloropsis thielavoides</i>	0.03	Intact thiamine
<i>Endomyces vernalis</i>	0.33	Pyrimidine
<i>Mucor ramannianus</i>	2.0	Thiazole
<i>Saccharomyces cerevisiae</i>	2.0	Pyrimidine and thiazole
<i>Staphylococcus aureus</i>	5.0	Pyrimidine and thiazole
<i>Salmonella gallinarum</i>	2.5	Pyrimidine and thiazole
<i>Neurospora crassa</i>	Greater than 4,000	None
<i>Escherichia coli</i>	Greater than 20,000	None
<i>Clostridium butylicum</i>	Greater than 20,000	None
<i>Lactobacillus arabinosus</i>	Greater than 400	None
<i>Lactobacillus casei</i>	Greater than 50,000	None
<i>Lactobacillus delbruckii</i>	Greater than 50,000	None
<i>Lactobacillus mesenteroides</i>	Greater than 50,000	None
<i>Lactobacillus pentoaceticus</i>	Greater than 50,000	None
<i>Streptococcus lactis</i> R	Greater than 50,000	None
<i>Propionibacterium pentosaceum</i>	Greater than 50,000	None
Hemolytic streptococcus H69D	Greater than 40,000	None

* Amounts are expressed in terms of pure pyriethamine. Compare footnote 3 of this chapter.

Antagonism between thiamine and pyriethamine in enzyme systems

The antagonism between thiamine and pyriethamine can be demonstrated *in vitro* in the absence of growing cells in an enzyme system. The system which converts thiamine to cocarboxylase has been observed thus to be affected (471). For example, when 10 gammas of the vitamin were used as substrate, 3 milligrams of pyriethamine reduced by 50 per cent the amount of cocarboxylase measurable after the enzymic reaction. Five milligrams of the analog prevented the appearance of any cocarboxylase. The enzyme system used in these experiments was the one found in chicken blood and was chosen because it forms relatively large amounts of cocarboxylase even when the tissue is derived from normal animals (472). In other tissues, synthesis has been observed only with preparations from thiamine-deficient animals (473). Even

then, the coenzyme is produced only in sufficient amount to restore the normal level, and excess thiamine added to such preparations does not result in further synthesis. Chicken blood, on the contrary, will produce about 60 gammas of cocarboxylase per 100 cubic centimeters, even when the tissue is taken from normal birds. The activity was found associated with the cellular components, but intact cells were not essential to the synthetical process, because washed cell suspensions fragmented in distilled water, or by additions of toluene, retained the ability to form cocarboxylase from added thiamine.

In assessing the point of action of pyrithiamine in such an enzymic process it must be remembered that several discrete reactions are involved in the measurement of the activity of the enzyme system. Thus, after the formation of cocarboxylase has taken place, the amount of it can be measured only by means of a series of enzymic processes. The synthesizing preparation is destroyed by heating, and the soluble cocarboxylase is then allowed to combine with apocarboxylase derived from yeast. Pyruvate ions are then added, and the extent of decomposition of them serves as a measure of the concentration of cocarboxylase in the system. When pyrithiamine is used to influence the first of these reactions, it may persist along with any cocarboxylase formed and may influence the succeeding processes. Conceivably it could be phosphorylated just as thiamine is, and the resulting pyrithiamine pyrophosphate might then retard the combination of thiamine pyrophosphate with apocarboxylase. Unchanged pyrithiamine might also interfere with the union of thiamine pyrophosphate and apocarboxylase. However, in the study mentioned above, this latter possibility was excluded, because pyrithiamine added to cocarboxylase did not retard its combination with the apoenzyme. Although the analog appeared to exert its effect on the synthesis of cocarboxylase, the possibility of formation and participation of pyrithiamine pyrophosphate could not be definitely excluded. However, no evidence for its formation could be found.

Oxythiamine, another antimetabolite of thiamine which will be discussed shortly, has also been reported to inhibit the synthesis of cocarboxylase from thiamine (431). A purified enzyme system derived from dried yeast was employed in this instance instead of the one from chicken blood cells. The same uncertainty about whether the analog was converted to a pyrophosphate, which then exerted a retarding influence, was inherent here, just as in the former case.

These findings have demonstrated that the effects of antimetabolites of thiamine in causing signs of thiamine deficiency in animals and

microorganisms can also be observed in enzyme systems free of growing cells. An interference with the synthesis of cocarboxylase, such as these analogs seem to cause, would aid in understanding the induction of thiamine deficiency in living things. However, this may not be the only point of interference with the action of the vitamin, or even the major one. It is merely the only one which has so far been studied. Other functions of thiamine in living cells conceivably may also be influenced by these antimetabolites. Nevertheless, all enzymic processes in which this vitamin acts as substrate are not so affected. For example, the system extracted from fish viscera which cleaves the thiazole portion from thiamine (238) is not inhibited by pyrithiamine (428, 429). Rather, the enzyme appears to attack the analog as it does thiamine.

Other structural analogs antagonistic to thiamine

After the demonstrations with pyrithiamine, investigations soon revealed that the structure of the metabolite could be altered in several other ways with consequent production of antimetabolites; e.g., the amino group in the 4-position of the pyrimidine ring was exchanged for an hydroxyl group (6), and the resulting compound (oxythiamine) was found capable of eliciting the signs of thiamine deficiency in mice. However, these signs were limited to inanition and failure of growth and did not include the pharmacological manifestations observable with pyrithiamine (431). In chickens also a state resembling thiamine deprivation has been produced with oxythiamine (474). In both species the effects could be prevented by increasing the amount of thiamine in the diet, but the competitive nature of the relationship with chickens was not so clear as in the case of pyrithiamine and thiamine.

Another antagonist to thiamine in laboratory animals was found when the methyl group in position 2 of the pyrimidine ring of the vitamin was replaced by a butyl group (7). An even more profound alteration of the structure such as the replacement of the entire pyrimidine ring of the metabolite with an amino-benzene ring led to the formation of a compound which competed with the vitamin in a specific enzyme reaction (the thiaminase of fish tissue) (8, 9). Structural alterations of the thiazole part of the molecule, such as the replacement of this ring system with an imidazole (10, 11), likewise have proved effective. Not all changes in the structure of the molecule, however, lead to antagonistic agents. For example, the replacement of the methyl group in position 2 by an ethyl radical yielded no antagonist, but rather a compound with some degree of vitamin activity (12).

Generality of the phenomenon

For nearly all the vitamins, for several hormones, for many amino acids, and for other important metabolites, structural analogs have been found which are capable of competing with, or of antagonizing the action of, these substances. The purpose of this monograph is not to provide a catalog of all these cases, because the detailed information can be found with greater ease in *Chemical Abstracts*, where it is accompanied by more specific and individual information than would be advisable to provide in the present book. Rather, this discussion has been planned to correlate the vast amount of such information, and more especially to attempt to draw from it some understanding of the causes and uses of the phenomenon. Let us embark on this course by reviewing briefly several of the facts about a few selected analogs of each of a number of important metabolites. We shall then have a basis on which to discuss principles, albeit not an encyclopedic knowledge.

Table 4 shows in a condensed form most of the types of inhibitory structural analogs which have been studied for a wide variety of metabolites. The references to the literature given for each analog or class of analogs are representative rather than exhaustive. For some metabolites it has seemed desirable to list the antimetabolites in separate tables by themselves. These supplement the information in Table 4. Furthermore, many of the cases require explanatory or qualifying statements, and these have been summarized in the remaining part of this chapter.

Antimetabolites of *p*-aminobenzoic acid other than derivatives of sulfanilamide

The structure of *p*-aminobenzoic acid may be altered in ways other than that which leads to the formation of the sulfonamide drugs, and many of the substances so produced can be shown to antagonize the action of the metabolite. Indeed, when *p*-aminobenzoic acid was recognized as an important metabolite as a result of Woods' work with the sulfonamides, it became apparent that some other widely used therapeutic agents likewise were analogs of this metabolite. For example, carbarsone was seen to be merely *p*-aminobenzoic acid in which the carboxyl group had been replaced by an arsonic acid radical, and the amino group had been converted to a urethane. With these newly recognized analogs some reversal of their biological effects could be demonstrated with the metabolite. Thus the toxic effects on rats of these arsenical drugs could be prevented by prior treatment of the animals with *p*-aminobenzoic acid (40-43). However, *p*-aminobenzoic

Table 4

Metabolites and structural analogs which have been found antagonistic to them

Metabolite	Analog	Structural Alteration	Biological System Affected	References
Acetic acid	Fluoroacetic acid	F for H	Acetic oxidase	13, 14
Adenine	Benzimidazole and derivatives	2 C for 2 N; side-chain alterations	Microorganisms, animals	15, 16, 17
	Triazolopyrimidines	N for C	Microorganisms	18
	Diaminopurine	NH ₂ for H	Bacteria	19
β -Alanine	β -Aminobutyric acid	CH ₃ for H	Yeast	20
	Propionic acid	H for NH ₂	Bacteria	21
	Asparagine	COOH for H; CONH ₂ for COOH	Yeast	22
α -Alanine	Glycine	H for CH ₃	Bacteria	23
Arginine	Canavanine	O for CH ₂	Bacteria	423
Aspartic acid	Hydroxyaspartic acid	OH for H	Bacteria	24
	Aspartophenone	C ₆ H ₆ for OH	Bacteria	25
Ascorbic acid	Glucoscorbic acid	Addition of CHO and optical inversion	Animals, liver enzymes	26, 27, 28, 29
<i>p</i> -Aminobenzoic acid	Sulfanilamide and derivatives	SO ₂ NH ₂ or derivative for COOH	Microorganisms	30, 31, 32, 33, 34, 35
	<i>p</i> -Aminobenzamide	CONH ₂ for COOH	Bacteria	36, 37
	Carbarsone and related arsenicals	As for C in a COOH group; derivatives of this	Microorganisms, animals	36, 38, 39, 40, 41, 42, 43, 433, 434, 435
	Phosphanilic acid	PO ₃ H ₂ for COOH	Microorganisms	44
	Heterocyclic acids, e.g., 6-aminonicotinic acid	N or S for C	Bacteria	45
	Ring-substituted PAB	Halogen or alkyl for H	Bacteria	45, 46, 47
	<i>p</i> -Aminoacetophenone and derivatives	COR for COOH	Bacteria	37, 48
	<i>p</i> -Nitrobenzoic acid	NO ₂ for NH ₂	Bacteria	49
Biotin	Desthiobiotin and derivatives	2 H for S	Microorganisms	50, 51, 52, 53, 54
	Biotin sulfone	SO ₂ for S	Microorganisms	51, 407
	Ureylene cyclohexyl aliphatic acids	2 C for S; and derivatives with shorter side chains	Microorganisms	55, 56
	Desthioisobiotin	Loss of S, geometric isomerism	Insects	57
	Ureylene tetrahydrofuryl aliphatic sulfonic acids	O for S, SO ₃ H for COOH	Microorganisms	58, 406
	Homobiotin	Addition of —CH ₂ —	Microorganisms	407, 408
Choline	Triethyl choline	3 ethyls for 3 methyls	Frog muscle and mice	59
Coccarboxylase	Thiamine-thiazole pyrophosphate	Loss of pyrimidine portion	Carboxylase	60
<i>Cis</i> - or <i>trans</i> -crocetin dimethyl ester	<i>Trans</i> - or <i>cis</i> -crocetin dimethyl ester	Geometric isomerism	Algae	61
Cytidine	Adenosine	OH for H, loss of imidazole ring	<i>Neurospora</i> mutant	62
Desthiobiotin	2-Oxyimidazole aliphatic acids	H for CH ₃	Microorganisms	54

Table 4 (Continued)

Metabolites and structural analogs which have been found antagonistic to them

Metabolite	Analog	Structural Alteration	Biological System Affected	References
Folic acid	See Table 5			
Glutamic acid	Methionine sulfoxide	SOCH ₃ for COOH	Bacteria	63, 64, 65
	Hydroxyglutamic acid	OH for H	Bacteria	66
	N-alkylglutamines	N-Alkyl for OH	Bacteria	67
Guanine	Triazolopyrimidines	N for C	Bacteria	18, 484
	Benzimidazole	2 C for 2 N	Microorganisms	16
Histamine	Imidazole and derivatives	Elimination or substitution of part of the molecule	Smooth muscle, histamine shock in animals	31, 68, 69, 70, 71, 72, 73, 74, 75
	Benadryl	Opening of ring, O for N, alkylation of N and C	Smooth muscle, histamine shock in animals	76
	Pyribenzamine	Opening of ring, alkylation of N	Smooth muscle, histamine shock in animals	76
Hypoxanthine	Hydroxytriazolopyrimidine	N for C	Bacteria	18
Indoleacetic acid	Phenyl butyric acid	Elimination of N and shift of one C	Plants	77
Inositol	Skatyl sulfonic acid	SO ₃ H for COOH	Plants	401
	Hexachlorocyclohexane	6 Cl for 6 OH	Fungi, plants, pancreatic amylase	78, 79, 83, 402
Isoleucine	Leucine	Position isomerism of one CH ₃	Bacteria	80
Leucine	<i>d</i> -Leucine	Optical inversion	Bacteria	81
Lysine	Arginine	Guanidino for amino, elimination of CH ₂	<i>Neurospora</i> mutant	82
Methionine	Methoxinine	O for S	Bacteria	18
	Ethionine	CH ₃ for H	Bacteria and animals	84, 85, 403
Nicotinic acid (or amide)	Norleucine	CH ₂ for S	Bacteria	86
	Pyridine-3-sulfonic acid or amide	SO ₃ H for COOH	Microorganisms, animals	87, 88, 89, 90, 91, 92, 93
	3-Acetylpyridine	COCH ₃ for COOH	Animals, not in microorganisms	48, 94
	5-Thiazole carboxamide	S for CH=CH	Certain bacteria	91, 95
Pantothenic acid	See Table 6			
Phenylalanine	β -Hydroxyphenylalanine	OH for H	Bacteria	96
	Thienylalanine	S for CH=CH	Microorganisms, animals	96, 97, 98, 25, 400, 405
	Furylalanine	O for CH=CH	Microorganisms	25
	Halogenated phenylalanines	Halogen for H	Microorganisms	99
Pimelic acid	2,4-Dichlorosulfanilidocaproic acid	Dichlorosulfanilide for COOH	Biotin independent microorganisms	314
Porphyryns such as hematin and protoporphyrin	Porphyryns lacking vinyl groups		Bacteria	100
Pteroylglutamic acid	See Table 5			

Table 4 (Continued)

Metabolites and structural analogs which have been found antagonistic to them

Metabolite	Analog	Structural Alteration	Biological System Affected	References
Pyridoxine	Desoxypyridoxine	H for OH	Animals, microorganisms	101, 102, 103, 104, 146, 200, 201, 419
	2-Ethyl-3-amino-4-ethoxymethyl-5-amino-methyl pyridine	CH ₃ for H, NH ₂ for OH, Et for H	Microorganisms	104
Riboflavin	6,7-Dichlororiboflavin	2 Cl for 2 CH ₃	Microorganisms	105, 106
	Isoriboflavin	Shift in position of CH ₃	Animals, not bacteria	107, 108
	Corresponding phenazine	2 C for 2 N, 2 NH ₂ for 2 OH	Microorganisms, animals	109, 110
	Galactoflavin	Dulcetyl for ribityl	Animals	111
	Lumiflavin	CH ₃ for ribityl	Bacteria	1, 110, 112
	Araboflavin	Inversion of position of OH	Animals	113
Succinic acid	Malonic acid	Loss of CH ₂	Succinic oxidase	114
	Sulfonated succinic acid	SO ₃ H for H	Succinic oxidase	115
Testosterone	Estradiol	Benzene ring for cyclohexane ring, loss of CH ₃	Animals	116, 117, 118, 119
Thiamine	Pyritiamine	CH=CH for S	Animals, microorganisms	2, 5, 120, 121, 122, 123, 124, 125
	Oxythiamine	OH for NH ₂	Animals, fish thiaminase	6, 431, 474
	Butylthiamine	Butyl for CH ₃	Animals	7
	Aminobenzylmethylthiazolium chloride	2 C for 2 N, loss of side chains	Fish thiaminase	8, 9
Thymine	5-Substituted dioxypyrimidines	NO ₂ or Br or NH ₂ or OH for CH ₃	Bacteria	19, 126
	2,4-Diamino or dithiothymine	NH ₂ or SH for OH	Bacteria	19, 126
α -Tocopherol	α -Tocopherol quinone	Opening of ring by addition of O	Animals	127
Thyroxine	Ethers of diiodotyrosine	<i>p</i> -Nitrobenzyl or <i>p</i> -nitrophenylethyl or benzyl for <i>p</i> -hydroxydiiodophenyl	Tadpoles	128
Tryptophane	Indole acrylic acid	Loss of NH ₃	Bacteria	129
	Naphthylacrylic acid	Loss of NH ₃ , C=C for N	Bacteria	130
	Styrylacetic acid	Loss of NH ₂ , substitution of aliphatic unsaturated side chain for pyrrol ring	Bacteria	130
	Methyltryptophanes	CH ₃ for H	Bacteria	131, 132
	Benzothienylalanine	S for N	Bacteria	133
Tyrosine	Indole	Loss of side chain	Bacteriophage plus bacteria	134
	3-Fluorotyrosine	F for H	Rats	135

Table 4 (Continued)

Metabolites and structural analogs which have been found antagonistic to them

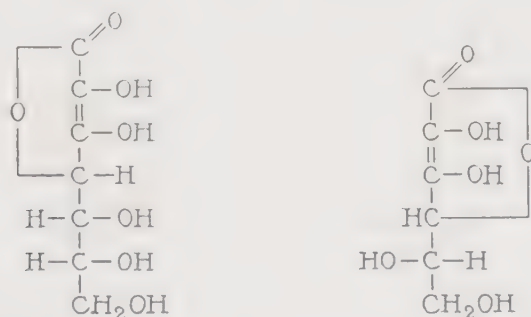
Metabolite	Analog	Structural Alteration	Biological System Affected	References
Uracil	Barbituric acid Thiouracil	OH for H S for O	Bacteria Bacteria, plant seed germination	136 137, 404
Vitamin K	Dicoumarol and derivatives	O for C, side-chain alterations	Animals	202, 203, 204, 206, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221
	Iodinin	2 N for 2 C, side-chain alterations	Bacteria	222
	α -Tocopherol quinone	2 CH ₃ for benzene ring	Animals	223
	2,3-Dichloronaphthoquinone	2 Cl for alkyl side chains	Microorganisms	208, 209
	2-Substituted-3-hydroxynaphthoquinones	OH for H, change in alkyl substituent	Animals, not bacteria	207
	Methoxynaphthoquinone	OCH ₃ for CH ₃	Microorganisms	210

acid usually could not save an animal if the toxic manifestations of the drug had appeared before the metabolite was administered. Furthermore, in a few cases it was possible to antagonize the action of the drugs on microorganisms (434) with the metabolite, but this could not always be done and in several species difficulty in the demonstration was experienced. The interference of the metabolite with these arsenical drugs therefore lacks the mathematical precision and almost universal applicability which characterize the relationship in the sulfonamides. Prior work on the mode of action of arsenic compounds on trypanosomes and spirochetes had led to the conclusion that therapeutic effect on diseases caused by these organisms was only exerted by arsenoxide derivatives (433). Pentavalent arsenicals were apparently reduced in the host before they attacked the parasite. The effect which they then had has been attributed to interaction of the arsenic with essential thiol groups. In view of these findings it is uncertain what interpretation should be placed on the antagonism to *p*-aminobenzoic acid which can be demonstrated in other types of organisms. Some investigators (436) are of the opinion that substances such as atoxyl act on bacteria by antagonizing *p*-aminobenzoic acid but attack trypanosomes by combining with essential thiol groups.

Besides these examples in which the structural change has been principally in the carboxyl group, either the ring system itself or the groups attached to it may be altered in such a way as to produce agents which interfere with bacterial growth competitively with *p*-aminobenzoic acid. Some of these analogs have been of use in tracing metabolic reactions, although none has thus far proved to be of lasting value as a therapeutic agent. For example, the exchange of the benzene ring of the metabolite for other ring systems such as thiophene or furane gave bacteriostatic compounds (45). Similarly, replacement of the hydrogen atom at position 2 of the benzene ring by chlorine or alkyl groups gave active compounds (45, 46, 47). The replacement of this same hydrogen atom with an hydroxyl group to yield *p*-aminosalicylic acid provided a substance which was rather toxic to tubercle bacilli (144) and which has found some application in attempts at therapy of tuberculosis.

Structural analogs antagonistic to other water-soluble vitamins

Ascorbic acid analogs. When glucoascorbic acid in relatively large amounts (5 per cent of the diet) is fed to mice maintained on a highly purified ration, a disease is produced (26) with manifestations very



Glucoascorbic acid

Ascorbic acid

Figure 3. Structures of ascorbic acid and glucoascorbic acid.

similar to those seen in scurvy of men and guinea pigs. The hemorrhages in the gingivae, in the joints of the long bones, and in the skin, the edema of the ankles, the decalcification of the bones, all of which may be seen in scorbutic men or guinea pigs, are present in these mice. In addition, there is extensive diarrhea. Unlike the situation in scurvy, the teeth do not become loosened or affected histologically. The structural resemblance of glucoascorbic acid to ascorbic acid may be seen in Figure 3. Scurvy as a dietary deficiency disease does not occur in mice, so that it is impossible to compare directly the condition provoked by the analog to that of a lack of the metabolite in this species. A further

difficulty is that simultaneous administration of ascorbic acid does not prevent or cure the disease caused by feeding the analog. Nevertheless, the similarity of the manifestations of disease caused by glucoascorbic acid to those seen in scurvy in other species has suggested to some investigators that the analog interferes with the action of the metabolite.

Although, in mice and in rats, ascorbic acid did not overcome the effects of glucoascorbic acid, a constituent of natural foodstuffs was found to do so. This substance was present in plant materials such as grass and cabbage and to some extent in liver (26, 412). It was clearly different from ascorbic acid because it was not destroyed by temperatures of 120°C. and because it differed in solubility behavior from the vitamin. Furthermore, as just indicated, ascorbic acid would not overcome the effects of glucoascorbic acid in these two species, whereas the unknown substance would. Because of the presence of this antagonist in natural foodstuffs, it was necessary to use diets compounded from pure substances rather than stock rations in studies with glucoascorbic acid. When large amounts of glucoascorbic acid were added to stock rations, signs of toxicity still could be produced, but for such a demonstration 10 per cent or more of the diet must be made up of glucoascorbic acid. The experiments with highly purified rations in which antagonism was demonstrated were performed with half of this amount or less. Even then, however, the required dose was large.

In weanling guinea pigs fed an adequate ration composed of purified substances, glucoascorbic acid produced a disease which was prevented by additions of small amounts (1 milligram per day) of ascorbic acid (27). Thus, in this species, an antagonism between the vitamin and its structural relative appeared. However, the condition provoked by glucoascorbic acid was not typical of vitamin C deficiency, because the tooth changes and alteration of serum phosphatase levels which are seen in scorbutic guinea pigs were not found in the disease caused by glucoascorbic acid (27, 29).

Because of the frequent inability of ascorbic acid to overcome the effects of glucoascorbic acid, a difference of opinion has arisen about whether or not it is an antimetabolite. The original investigators of its action on mice entertained the idea that it might be causing signs of ascorbic acid deficiency in these animals because of the resemblance of the condition which it produced to the signs of scurvy in species susceptible to that disease. They pointed out, however, that the failure of ascorbic acid to counteract it was difficult to understand in terms of such an hypothesis. The demonstration that, in guinea pigs fed a highly purified ration, the vitamin would, when fed in small amounts, antagonize the action of glucoascorbic acid seemed to favor the opinion

that the analog was actually an antimetabolite of ascorbic acid. A subsequent study sponsored the divergent opinion (412) that the toxicity of glucoascorbic acid was to be attributed to the diarrhea which accompanies its use. Although the results with rats were rather similar to those in mice, an experiment with two guinea pigs showed that, when ascorbic acid constituted 10 per cent of the food, glucoascorbic acid was still harmful to the animals, provided that large amounts were fed. Although this experiment was believed to prove that glucoascorbic acid was not an antimetabolite of ascorbic acid, the conditions were significantly different from those used in the first study, and therefore the two sets of findings do not necessarily conflict. In the experiment with the two guinea pigs, an amount of ascorbic acid which by itself was toxic (10 per cent of the food) was employed and the basal diet was a stock ration. Previous work had emphasized the need for a highly purified ration and the use of moderate amounts of ascorbic acid.

A third study also led to the opinion that glucoascorbic acid was not an antimetabolite of ascorbic acid (29). Here too a diet of natural foodstuffs was used so that the results are not directly comparable to those of the first study. However, glucoascorbic acid added to such a ration did not bring about the histological changes seen in the teeth of scorbutic guinea pigs. Neither did its addition to a highly purified diet cause the change in serum phosphatase which is found in scurvy.

So far as present evidence goes it seems to indicate that glucoascorbic acid, when added to a highly purified ration, causes in guinea pigs a disease which is not exactly like scurvy but has some similarities to it. This condition can be prevented by small amounts of ascorbic acid, but if toxic quantities of the vitamin are used, or if a stock ration is employed, complications are introduced which make it no longer possible to observe such antagonism. In mice and in rats, the condition which this analog elicits is not preventable by the vitamin, but is overcome by an unknown constituent of natural foodstuffs.

In enzyme systems as well as in intact animals, the action of glucoascorbic acid has been observed. For example, the oxidation of tyrosine by liver slices appears to be a process involving ascorbic acid. Glucoascorbic acid inhibited this reaction (28), and the inhibition was relieved with ascorbic acid.

Aside from glucoascorbic acid, no other structural analog of ascorbic acid has been found to give evidence of antagonizing the metabolite. For example, araboascorbic acid added to a highly purified ration was not toxic to mice at a level of 10 per cent (26).

The situation with glucoascorbic acid has been discussed in some detail because it illustrates a rather common finding. Quite frequently

an analog may call forth signs of a deficiency disease, but the metabolite to which it is related may not be capable of reversing these manifestations. After the factual evidence has been presented, this phenomenon will be discussed in detail in a subsequent chapter. Not uncommonly, the metabolite will overcome the effects of the analog in some species, whereas it fails to do so in others. However, the inability of the metabolite to nullify the effects of the analog has caused many investigators to reject the idea of a metabolic involvement of one substance with the other. This has been true for glucoascorbic acid and ascorbic acid (29, 412).

Biotin analogs. A variety of changes in the structure of biotin has yielded agents which will inhibit the growth of selected species of

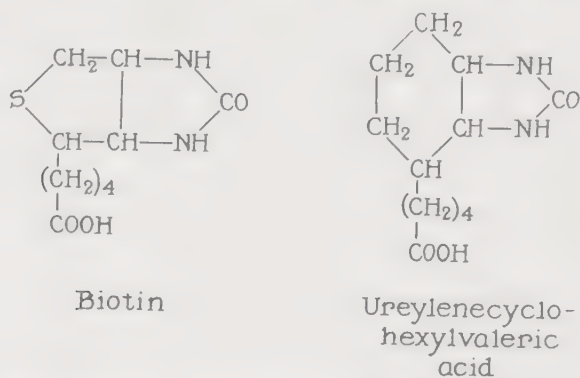


Figure 4. Structures of biotin and of ureylenecyclohexylvaleric acid.

microorganisms, and this effect has been overcome by increasing the biotin content of the medium. For example, the introduction of a cyclohexane ring in place of the thiophane ring of the vitamin (see Figure 4), or the elimination of the sulfur atom, or its oxidation to a sulfone (50-56) will give such inhibitory analogs. A similar result is achieved by replacement of the carboxyl group of the vitamin by a sulfonic acid radical and the simultaneous exchange of a sulfur in the thiophane ring for an oxygen atom. The next higher homolog of biotin, viz., homobiotin, which differs from it only by having an extra methylene in the side chain, has been reported to inhibit the growth of some yeasts in competition with the vitamin but to replace biotin as a growth factor for other species (407, 408). However, not all structural analogs of this vitamin show properties of an antimetabolite. For example, the thiazole relatives appeared to be inert (478). No extensive study of the effects of these compounds on animals has been recorded.

Choline analogs. The only structural analog yet found to behave antagonistically to choline is the triethyl derivative, the toxicity of

which for mice is overcome by an equal weight of choline (59). The same compound also interferes with the contraction of isolated frog muscles, and this effect is antagonized by choline. However, this analog can assume some of the functions of the metabolite in animals because, when fed, it is incorporated into the phosphatides. The same is true of arsenocholine, the analog in which arsenic has replaced the nitrogen of the metabolite (145).

Folic acid or pteroylglutamic acid analogs. A large number of structural analogs of this vitamin have been made and shown to inhibit the growth of certain species of microorganisms, and also to call forth the signs of folic acid deficiency in animals such as mice, rats, chickens, pigs,

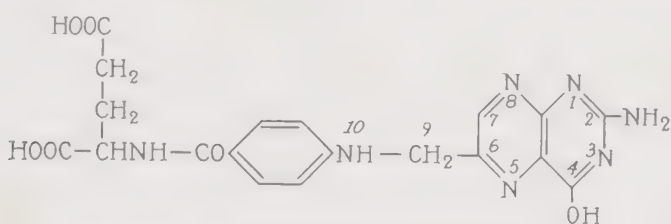


Figure 5. Structure of pteroylglutamic acid.

monkeys, and men. In most cases the inhibition of microbial growth which these substances cause is reversible with folic acid, but, with some of them, the signs of deficiency of the vitamin which they produce in higher animals are practically irreversible by pteroylglutamic acid.⁴ With few exceptions all these analogs affect only those bacteria which require folic acid in the medium and do not act against other species unless the agents are in high concentration. The structure of the vitamin is shown in Figure 5. Replacement of the hydroxyl group in position 4 of the pteridine ring by an amino group gives a highly potent inhibitor of growth of bacteria. This analog has sometimes been called aminopterin. The same compound in very small doses will call forth typical signs of folic acid deficiency in a wide variety of higher and lower animals but usually in these cases the disease which it causes cannot be prevented or cured with the vitamin.⁴ Some investigators have been able to protect mice and rats from the harmful effects of this com-

⁴ Some difference of opinion has appeared about the ability of folic acid to antagonize the action on animals. Some have reported that very large amounts of the vitamin given prior to the administration of the analog will counteract the latter. Furthermore, other metabolites believed to be involved in the functioning of folic acid can reverse the toxicity of these analogs. Thus, the effects of 4-aminopteroylglutamic acid on bacteria and higher animals may be prevented with the citrovorum growth factor (folinic acid) (413). The latter metabolite is apparently N-formyltetrahydropteroylglutamic acid (479) or an isomer of this.

pound when exceedingly large doses of folic acid are given simultaneously (414, 415). Other changes in the substituents of the pteridine ring system have also been found to yield active inhibitors both of bacterial multiplication and of the growth and normal functioning of animals. Thus the replacement of the hydrogen atom in positions 7 or 9 by a methyl group has been found effective. Substitution of methyl groups or of oxygen atoms for the hydrogen in the methylene bridge between the two ring systems gives agents which are quite potent. The pyrimidine portion of the pteridine ring system may be exchanged for a benzene ring in order to obtain an active inhibitor of the growth of folic acid-requiring bacteria. Likewise, the glutamic acid residue of the vitamin may be replaced by an aspartic acid residue or by certain other

Table 5

Some structural analogs antagonistic to pteroylglutamic acid

Structural Alteration *	Organisms Affected	Reversing Effect of Folic Acid	References
α -CH ₃ for H (α = 7 or 9)	Bacteria, chickens, rats, mice, swine, insects	Competitive	148, 149, 150, 151, 152, 153, 155
10-CH ₃ for H	Bacteria	Competitive	156
10-CH ₃ for H, loss of glutamic	Bacteria	Competitive	156
4-NH ₂ for OH	Bacteria, rats, mice, chickens, guinea pigs, insects, humans	Present in some bacteria, poor or absent in animals	154, 155, 157, 158, 159, 160, 161
4-NH ₂ for OH, 10-CH ₃ for H	Bacteria, chickens, rats	Poor or absent in animals	162
4-NH ₂ for OH, 6-phenyl for amino-benzoylglutamic, 7-phenyl for H	Bacteria, not animals	Present	163, 164
6-Phenyl for amino-benzoylglutamic, 7-phenyl for H	Chickens, not bacteria	165
7-OH for H, 9-O for H	Bacteria, rats	Competitive	166
Aspartic for glutamic	Bacteria, rats, mice, chickens	Competitive	167
Quinoxaline for pteridine	Bacteria, not rats	Competitive	166

* Numbers refer to the positions in the pteroylglutamic acid molecule at which the change was made.

amino acid residues in order to achieve antagonistic agents. Finally, combinations of these changes may be made in order to arrive at more active agents, or ones which have a desired selectivity of action on different organs or tissues. The information is catalogued and summarized in Table 5. A review which deals in detail with the various folic acid analogs has appeared (147). It seems probable that many more agents of this class will be investigated because of their ability to produce a desirable pharmacological effect, namely, leucopenia. This effect is a common manifestation of folic acid deficiency in higher animals.

Inositol analogs. Some evidence indicates that the highly effective insecticide, the gamma isomer of hexachlorocyclohexane, is an antagonist of inositol. The structural relationships are shown grossly in Figure 6. Although the exact spatial arrangement of inositol has been established, that of the chloro compound is uncertain. The evidence that these two structurally similar compounds are antagonistic is that the inhibition of growth of fungi which is caused by hexachlorocyclohexane can be reversed in some cases with inositol (78). There are many species, however, in which this is not true (168, 402). The effects of the analog on mitosis in onion roots can be overcome with the metabolite (79). Furthermore, the chloro analog is said to inhibit the action of pancreatic amylase, and inositol is said to overcome this effect (83). However, these claims have been challenged (475). In considering a possible antagonism between these compounds, one should note that conflicting claims have been abundant in the work which has been done, so that it is difficult to conclude with certainty about the existence or the nature of the antagonism. Possibly one reason why difficulty is experienced in demonstrating the antagonism in all species is that inositol is water soluble and not at all taken up by fat solvents, whereas the chloro analog is fat soluble and only very slightly so in water. If a biologically active but fat-soluble derivative of inositol were available, the antagonism between it and hexachlorocyclohexane might be demonstrated in many more species.

Some of the confusion about the relationship of hexachlorocyclohexane to inositol may have been resolved by the observation that

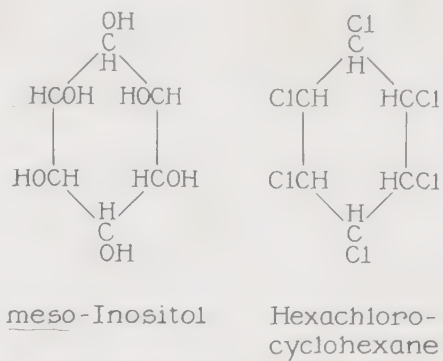


Figure 6. Structures of *meso*-inositol and of hexachlorocyclohexane.

inositol-requiring mutants of the fungus *Neurospora* allowed demonstration of the antagonism, whereas inositol-synthesizing strains did not. Both forms were affected in an irreversible fashion by sufficiently high concentrations of the analog, and therefore the antagonism by the metabolite in the inositol-requiring strain could be demonstrated only over a limited range. The inositol-requiring forms showed a characteristic morphological pattern when they grew in an inositol-deficient medium. This type of growth was likewise initiated by the analog and was made to revert to the normal pattern by increasing the inositol concentration (480). This failure to achieve reversal of the action of the antimetabolite in organisms which do not show a nutritional need for the metabolite has been found with glucoascorbic acid (related to ascorbic acid (26, 27)) and with phenylpantothenone (analogous to pantothenic acid (169)).

Nicotinic acid analogs. Relatively few analogs of this vitamin have been found capable of antagonizing its action. Pyridine-3-sulfonic acid

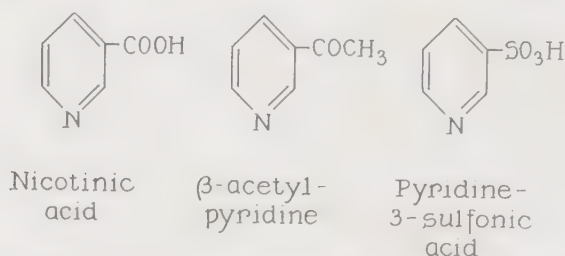


Figure 7. Structures of nicotinic acid, β -acetylpyridine and pyridine-3-sulfonic acid.

and its amide will inhibit the growth of selected strains of nicotinic acid-requiring bacteria (87-93), but the concentrations needed are large, and, although nicotinic acid or its amide will usually reverse the inhibition competitively, such diverse substances as ferrous ions as well as the vitamin have been reported to do likewise. In animals such as the mouse fed adequate, but highly purified diets, even large amounts are innocuous (93), but in nicotinic acid-deficient dogs it intensifies the deficiency (92). Judged by its failure to inhibit growth, β -acetylpyridine, on the other hand, does not seem to cause nicotinic acid deficiency in microorganisms but does produce typical signs of deficiency in animals such as mice, dogs, or chicken embryos (92, 94, 390). If the metabolite is given in large amounts before the analog is administered, the effects of the latter are prevented competitively and completely. However, once the analog has produced its effect, the animals cannot be saved by giving the metabolite. The effects of acetylpyridine have been observed on perfused hearts of cats (416), and the antagonism of its pharmacological action by nicotinamide has been studied. Here,

however, the order of addition of vitamin and analog to the tissue did not seem to be crucial. The structural resemblance of metabolite and analogs may be seen in Figure 7.

Pantothenic acid analogs. A large number of structural analogs of pantothenic acid have been found to inhibit the growth of microorganisms. Nearly all of them affect only those species which require pantothenic acid in the medium. A few, such as phenyl pantothenone and derivatives of it (169) are active against a wide variety of bacteria

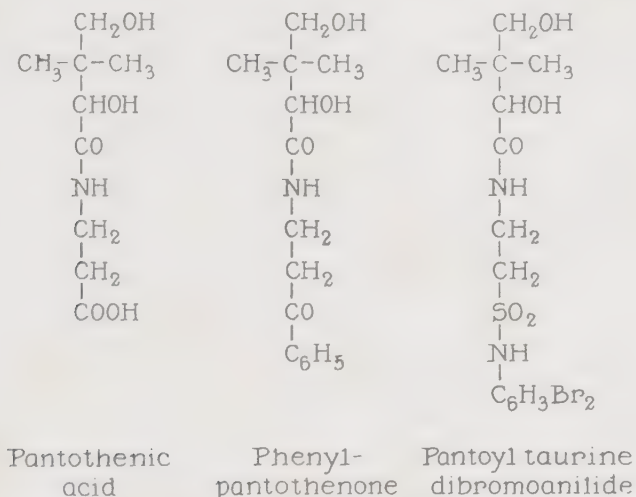


Figure 8. Structures of pantothenic acid, phenyl pantothenone, and pantoyltaurine dibromoanilide.

and yeasts, irrespective of pantothenic acid requirements. However, almost without exception,⁵ the pantothenic acid analogs do not cause the appearance of signs of pantothenic acid deficiency in animals (93, 197),⁶ and it is probably for this reason that some of them have shown promise as therapeutic agents against infectious diseases. Phenyl pantothenone and the halogenated anilides of pantoyltaurine are best known in this regard. The structures of the metabolite and of two of these inhibitory analogs are shown in Figure 8.

The types of inhibitory analogs of this vitamin fall into two groups: (1) those in which the β -alanine part of the molecule has been changed,

⁵ γ -Methylpantothenic acid is an exception because it appears to cause pantothenic acid deficiency in rats (170).

⁶ Snell et al. (417) concluded that pantoyltaurine produced pantothenic acid deficiency in weanling mice. They based this opinion on their findings that growth ceased after 4 weeks of administration of the analog. However, since weanling mice are mature in 3 or 4 weeks, the cessation of growth was not a critical test. Other investigations (93, 418) led to the conclusion that this analog did not produce a deficiency disease in animals.

and (2) those in which the alteration has been in the pantoyle moiety. A few inhibitory analogs have both parts of the molecule altered. In general, the compounds realized by changing the β -alanine portion have proved to be the most active antagonists of the metabolite, but an exception to this is found in γ -methylpantothenic acid, in which a methyl group has been added to the pantoyle residue, with the consequent formation of a rather potent antimetabolite (170). Among the analogs in which the β -alanine residue has been altered, pantoyletaurine (thiopanic acid) has shown activity against hemolytic streptococci and some other pantothenic acid-requiring bacteria (171–184). The structural change here is the replacement of a carboxyl group by a sulfonic acid radical. It does not hamper the growth of bacteria which do not need an external source of the vitamin, and, indeed, it does not prevent the multiplication of all species of pantothenic acid-requiring organisms. Because pantoyletaurine was one of the first antimetabolites to be predicted and synthesized with a specific view to its use as an antagonist to pantothenic acid, many studies have been carried out with it. For example, it was used to inhibit the utilization of pantothenic acid by resting streptococci (179, 182, 183) and to save experimental animals (rats) from an otherwise fatal infection with streptococci (174). Although this chemotherapeutic demonstration was only of theoretical interest because of the size of the dose needed, the halogenated anilides of this analog proved to be rather promising drugs when they were tested in experimental infections (185).

If the carboxyl group of the metabolite is replaced by a phenyl ketone, an antagonist to pantothenic acid is realized which is quite active in inhibiting the growth of a wide variety of microbial species (169). The compound, phenyl pantothenone, and some of its derivatives have shown promise as selective agents to suppress malarial infestations of animals and of man (17, 186). It also has found application in the investigation of metabolic reactions, such as in the competitive inhibition of coenzyme A synthesis from pantothenic acid in resting yeast cells (187).

Other modifications of the β -alanine portion of the vitamin have also been studied, such as the exchange of an alkyl or hydroxyalkyl group for the propionic acid radical (188–194). The number of possible analogs of this type is very large, and, of the numerous ones investigated, many have proved to be antagonists to the vitamin.

Turning to antagonists in which the pantoyle residue of the metabolite has been altered, one sees that only a few active compounds have been found. Many which have been tested have proved to be inactive. Among the active substances one can see examples of how great the

Table 6

Antimetabolites to pantothenic acid

Analog	Structural Alteration	Inhibition Index *			References
		<i>L. arab- inosus</i>	Yeast	<i>L. casei</i>	
<i>A. Alterations of β-alanine part</i>					
Pantoyltaurine (thiopicnic acid)	SO ₃ H for COOH	1,000	24,000	171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 194
Pantoyltaurine amide	SO ₂ NH ₂ for COOH	6,400	194
Pantoyltaurine anilides	SO ₂ NHC ₆ H ₅ for COOH	185
Phenylpantothenone	COC ₆ H ₅ for COOH	4,500	Inhibition not reversible	700	169, 198
Tolyl pantothenone	COC ₆ H ₄ CH ₃ for COOH	100	199
Chlorophenyl pantothenone	COC ₆ H ₄ Cl for COOH	230	199
α -Methylpantothenic	CH ₃ for H	1,000	190, 191
β -Methylpantothenic	CH ₃ for H	750	250	191
Pantothenol	CH ₂ OH for COOH	10,000	20,000	191
Methylpantothenol	CH(CH ₃)OH for COOH	50,000	100,000	191
α -Hydroxypantothenic †	OH for H	2,500	2,500	191
Pantoic hydrazide	NH ₂ for CH ₂ CH ₂ COOH	240	194
Monopantoylalkyldiamines	Alkyl diamine for β -alanine	around 100,000	194
Tolyl sulfone of pantothenic	SO ₂ C ₆ H ₄ CH ₃ for COOH	6,400	194
Anisyl sulfone of pantothenic	SO ₂ C ₆ H ₄ OCH ₃ for COOH	1,600	194
Pantoylpropylamine	CH ₃ for COOH	7,500	10,000	193
Pantoylbutylamine	C ₂ H ₅ for COOH	7,500	15,000	193
Pantoylheptylamine	(CH ₂) ₄ CH ₃ for COOH	4,500	4,250	193
Pantoylphenylethylamine	C ₆ H ₅ for COOH	40,000	10,000	193
<i>B. Alterations of pantoyl part</i>					
γ,γ -Dimethyl- δ -hydroxyvaleryl- β -alanine	CH ₂ CH ₂ for CHO	Inhibitory to streptococci but not reversed by pantothenic			175, 173
γ -Hydroxybutyryl- β -alanine	Loss of methyls and of α -hydroxy				
γ -Methylpantothenic †	CH ₃ for H	5,000	300	170
Salicylyl β -alanine	Hydroxybenzoic for pantoic	1,600	12,500	195, 196
<i>C. Alterations of both portions</i>					
β - δ -Dihydroxy- γ,γ -dimethylvaleryltaurine	Insertion of CH ₂ , SO ₃ H for COOH	Inhibitory to hemolytic streptococci			175, 173

* As will be explained in a succeeding chapter, the inhibition index represents the amount of antimetabolite required to overcome the effect of a unit weight of metabolite. The values in this table were calculated from the quantities required to produce maximal inhibition of growth. Since they were assembled from the results obtained in different laboratories the values are probably strictly comparable only where they arose from one investigator. Results with the same compound in different laboratories may vary somewhat because of differences in time of incubation and in basal media.

† Complete inhibition of growth could not be achieved with this compound.

‡ Nomenclature used for pantothenic acid derivatives is sometimes confusing because some investigators name the α -carbon atom of the entire molecule, whereas others take the α -carbon atom of the pantoyl residue as the α -carbon atom. γ -Methylpantothenic acid might more correctly be named ω -methylpantothenic acid.

change in structure can be in the formation of some antimetabolites. Thus, salicylyl β -alanine will inhibit the growth of yeast and this action can be reversed competitively by pantothenic acid (195, 196). In this antimetabolite, salicylic acid replaces α,γ -dihydroxy- β,β -dimethylbutyric acid (pantoic acid). Many of the analogs antagonistic to pantothenic acid are listed in Table 6. One can see from this table the relative effect on activity of graded and diverse changes in structure.

Pyridoxine analogs. If the hydroxymethyl group in position 4 of pyridoxine is replaced by a methyl group, an analog known as desoxy-pyridoxine is obtained (see Figure 9). This compound when given in

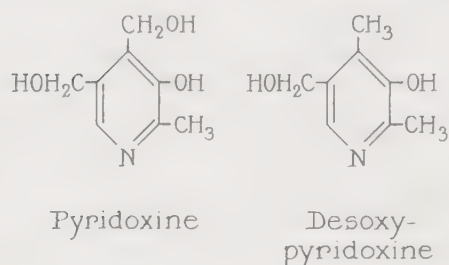


Figure 9. Structures of pyridoxine and of desoxypyridoxine.

very small doses is able to produce the signs of pyridoxine deficiency in chickens, rats, or mice, provided that these animals are receiving a ration suboptimal in this vitamin. The signs may be overcome by increasing the amounts of the vitamin. In the presence of adequate amounts of the metabolite, large doses of the analog are required to give an effect, and thus the action

is not competitive (101–104, 146, 200, 201, 419). Apparently the analog is phosphorylated in the animal, and this product then acts to prevent the attachment of pyridoxal phosphate to the apoenzyme of the amino acid decarboxylases (146).

If the criterion of activity is taken as inhibition of growth of pyridoxine-requiring yeast, then 2-ethyl-3-amino-4-ethoxymethyl-5-amino-methylpyridine is found to be an antimetabolite, the action of which is reversed by the vitamin (104).

Riboflavin analogs. Several types of structural alteration of riboflavin have led to the formation of compounds which are antagonistic to this vitamin. For example, replacement of the methyl groups in positions 6 and 7 by chlorine atoms yielded an analog which inhibited the growth of some species of microorganisms (105, 106). Shifting the position of the methyl group in position 7 of the vitamin to position 5 likewise gave a compound which was antagonistic to the metabolite. This was shown by the ability of the analog to cause signs of riboflavin deficiency in rats (107, 108), although apparently not in microorganisms.⁷ Substituents other than these methyl groups may be exchanged

⁷ The authenticity of isoriboflavin as an antimetabolite has been called in question by a recent observation (481) that, whereas the original sample showed some effect in rats fed suboptimal riboflavin, subsequent samples exhibited no activity.

with the resulting production of inhibitory analogs. Thus, when a galactose or an arabinose residue is substituted for the ribose residue in position 9, analogs capable of producing signs of riboflavin deficiency in animals (111, 113) are formed. Changes may also be made in the ring system in order to form antagonists of the vitamin. Thus, when the isoalloxazine ring system is replaced by a phenazine, and the oxygens in positions 2 and 4 are traded for amino groups, the analog so produced is active in antagonizing the action of riboflavin both in animals and

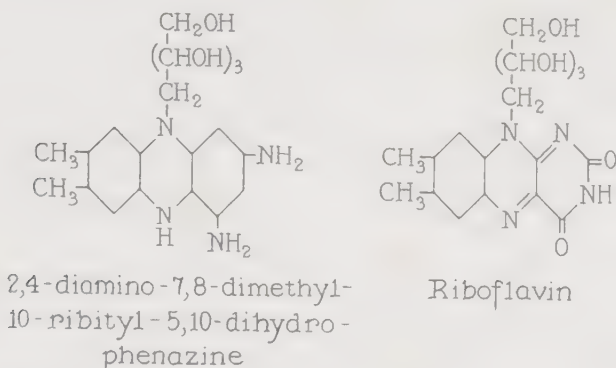


Figure 10. Structures of riboflavin and of its phenazine analog.

in riboflavin-requiring bacteria (109, 110), although the potency of the agent is rather low. Some of the structural resemblances of the vitamin to the analogs may be seen in Figure 10.

Structural analogs antagonistic to fat-soluble vitamins

Vitamin K analogs. Largely by chance a considerable number of structural analogs antagonistic to vitamin K have been discovered. The action of some of these compounds can be overcome with the vitamin. Furthermore, a few of them call forth in animals the characteristic aberration of blood clotting which is associated with avitaminosis-K. In no instance, however, does a clear case of competition between the vitamin and its analog exist such as can be demonstrated with thiamine and pyrithiamine, or with *p*-aminobenzoic acid and sulfanilamide, and for this reason it may be advisable to state briefly some of the experimental findings.

Rather extensive alteration of the structure of the vitamin by the replacement of the naphthalene ring system with a coumarin system, and also a considerable change in the alkyl side chain, leads one from vitamin K to dicoumarol, as can be seen in Figure 11. This coumarin derivative was first studied biologically because it was isolated from spoiled sweet-clover hay and identified as the causative agent of a

hemorrhagic disease which resulted when animals ate such hay (202). Since dicoumarol produced the fall in prothrombin level in the blood which had been regarded as a sign of vitamin K deficiency, this vitamin was tested as an antidote and found to protect against the toxic agent (203, 204). The structural resemblance of the vitamin to the poison was then recognized (205, 206) and, taken with the facts already recounted about the signs of toxicity and their prevention with vitamin K, sponsored the opinion that dicoumarol was an antimetabolite of this vitamin. The difficulty with this view is that large doses of the vitamin are needed to overcome the effects of the agent, and that amounts of

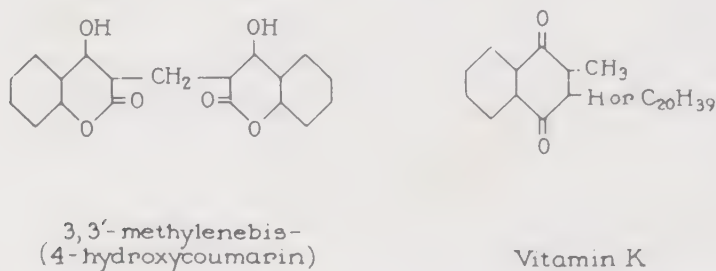


Figure 11. Structures of vitamin K and of dicoumarol (3,3'-methylenedis-(4-hydroxycoumarin)).

dicoumarol greater than about twenty-five times the minimal lethal dose cannot be counteracted by the vitamin. If dicoumarol were an "uncomplicated" case of an antimetabolite, one would expect that a minimal lethal dose of it could be rendered harmless by small quantities, say a few micrograms, of the vitamin. This would be expected, since only a minute amount of vitamin K is required to cure the deficiency disease resulting from its absence from the diet. Nevertheless, about 10 milligrams are needed to protect against a minimal lethal dose of dicoumarol. Since vitamin K active compounds show toxicity by themselves when these relatively enormous doses are given, the reason for the inability to overcome amounts of dicoumarol greater than about twenty-five lethal doses may be related to inherent toxicity of massive amounts of the vitamin. Dicoumarol is a weak inhibitor of bacterial growth, and its action in this regard is not overcome by vitamin K (420). This failure to demonstrate antagonism may be related likewise to toxicity of large amounts of the vitamin.

Empirical examination of many coumarins with substituents in a variety of positions revealed that those with long alkyl groups in position 3, and with an hydroxyl in position 4, possessed activity similar to that of dicoumarol (204). The structural resemblance of such substances to vitamin K is greater than is that of dicoumarol, but the com-

pounds are no more active in causing the signs of vitamin K deficiency. However, it should be pointed out that the derivative with a phytyl chain in the molecule has not been examined, and it is the phytyl group which is found in the vitamin.

The ring system of vitamin K may be altered in ways other than the conversion of it to a coumarin derivative, and some of the substances which are thus formed have an effect on animals similar to that of dicoumarol (206). For example, the sulfur or nitrogen analogs of dicoumarol have such an action.

The possible importance of preserving the long alkyl side chain of the vitamin, which was alluded to above, may be seen from the fact that 2-cyclohexylpropyl-3-hydroxy-1,4-naphthoquinone, which differs from the vitamin only in the nature of the side chains, causes the diminution in prothrombin and consequent hemorrhage in rats which are associated with avitaminosis-K (207), and does so in contradistinction to 3-hydroxynaphthoquinone which apparently does not.

The realization of a highly effective antagonist merely by changing the side chains of the naphthoquinone ring system, as well as the qualitative difference in biological effect which this causes, may be seen with 2,3-dichloro-1,4-naphthoquinone. This compound was developed empirically with no thought of its resemblance to a metabolite and was found highly poisonous to fungi (208). It differs from vitamin K only in that the alkyl side chains in positions 2 and 3 have been replaced by chlorine atoms. Its action in preventing the growth of fungi is overcome by minute amounts of the vitamin (209), and over a very limited range of concentration the antagonism is competitive in character. The difficulty is that only with small doses of the analog is it possible to overcome the inhibition of growth with the vitamin. Since the vitamin itself is rather toxic to fungi, one may seize upon an explanation of this failure, but the case would be much stronger if reversal of the toxic effects were possible over a greater range of concentration. Furthermore, the analog, even in large amounts, does not call forth in animals the signs of avitaminosis-K, and in this regard it differs sharply from several other substances already discussed.

The toxicity to fungi of certain analogs of vitamin K has apparently been put to use by some plants. An antibiotic agent from garden balsam has been isolated and identified as 2-methoxy-1,4-naphthoquinone (20). In a few instances, but not in all, the antifungal action of this substance may be overcome with the vitamin.

Chromobacterium iodinum similarly has produced an analog of vitamin K by altering both the ring system and the substituents and has thus formed a substance which inhibits the growth of other bacteria



(222). This substance is apparently a dihydroxyphenazine oxide. The inhibition of the growth of streptococci, which it causes, is overcome with small amounts of vitamin K, and also by some naturally occurring compounds without vitamin activity. The structural analogy of this compound to vitamin K may be seen if one replaces the carbon atoms in positions 1 and 4 of the vitamin with nitrogen atoms and modifies the alkyl side chains in positions 2 and 3 in order to obtain an hydroxy-phenyl ring fused to the prior ring system at two points.

Vitamin E or tocopherol analogs. Whether the only inhibitory structural analog of tocopherol should be discussed under vitamin K

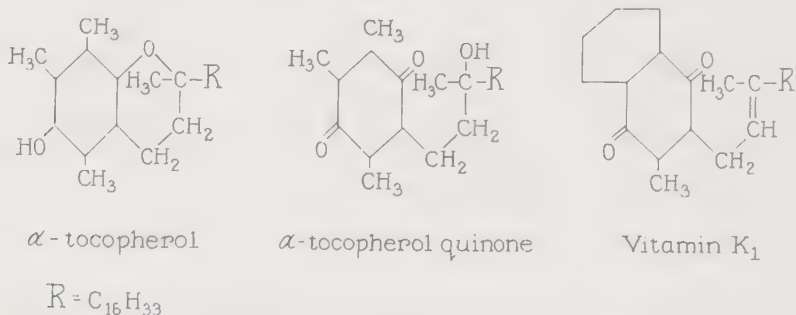


Figure 12. Structures of α -tocopherol, α -tocopherol quinone, and vitamin K.

or under tocopherol is uncertain. When the ring system of vitamin E is broken by oxidation, α -tocopherolquinone is formed. Large doses of this substance cause the interruption of pregnancy in mice at about the fourteenth day, and the subsequent resorption of the embryos just as is seen in tocopherol deficiency of this species (223). It would thus seem that α -tocopherol quinone causes the characteristic signs of vitamin E deficiency. The difficulty with this interpretation is that tocopherol will not prevent the condition elicited by the analog, but, on the other hand, vitamin K will do it. The quinone is an analog of both vitamins at the same time, as one can see in Figure 12.

Structural analogs antagonistic to amino acids

Although many examples of antagonism between pairs of naturally occurring amino acids of closely analogous structure have been described, many of them are complicated by the fact that frequently a variety of amino acids, the closely analogous as well as the distantly related ones, will overcome the toxic manifestations of a given compound. Although this same complexity likewise affects the demonstrations with synthetic structural analogs of several amino acids, the phenomena may be clarified if we limit the present discussion to these latter cases, and reserve for a future chapter any attempt to understand

the more involved relationships between pairs of naturally occurring and mutually antagonistic amino acids.

The complexity which besets any study of even the synthetic structural analogs of the amino acids is well illustrated by one of the first explored, namely that of the α -aminosulfonic acids (224). A number of these sulfonic acids were prepared, each of which was the same as some natural amino acid except that the carboxyl group of the metabolite was exchanged for a sulfonic acid radical. These substances were found to inhibit the growth of some bacteria, and this effect was abolished by the presence of the corresponding natural amino acid. However, it was also abolished by other amino acids less closely related to the inhibitor. We shall see in a subsequent chapter how this behavior may possibly arise from the ability of the organism to interconvert many of the natural amino acids, but, at best, the phenomenon lacks clarity of explanation.

Tryptophane analogs. The structure of tryptophane has been altered in a great many ways with the attendant formation of analogs which behave antagonistically to it. Most of the biological testing has consisted of demonstrations that the analogs will inhibit the growth of bacteria. Fildes (129) and his collaborators (132) have examined many of these tryptophane antagonists and have shown that some probably inhibit one of the last stages in the synthesis of the amino acid, whereas others interfere with its utilization. Thus, indoleacrylic acid, which lacks only a molecule of ammonia to become tryptophane, probably competes with some unknown precursor of similar structure and thus prevents the synthesis of the amino acid. On the other hand, a substance such as 5-methyltryptophane probably competes directly with the amino acid and thus interferes with its utilization, i.e., its conversion to other substances. This latter function can be seen from the effects of the methyl analog on the binding of bacteriophage to *Escherichia coli* (131). Tryptophane apparently acts as a link by means of which the bacteriophage is attached to the bacterial cell. In performing this function it may be displaced and thus rendered inert by the simultaneous presence of 5-methyltryptophane (225).

Some of the other kinds of tryptophane analogs which have been found to inhibit bacterial growth are listed in Table 4. In general, the phenomenon of competition of the metabolite with the antimetabolite stands out clearly with tryptophane analogs, because only this amino acid, and not others, reverses the toxic manifestations.

Phenylalanine analogs. By alteration of the structure of phenylalanine in a variety of ways a number of substances have been produced which compete with it and thus inhibit the growth of bacteria. The

replacement of one or two carbon atoms of the benzene ring by a nitrogen atom, or by an oxygen or a sulfur atom, has yielded antagonists of this amino acid. Compounds inhibitory of microbial growth may also be achieved by replacement of a hydrogen atom on the benzene ring with a fluorine or other halogen atom (99), or by substitution of a hydrogen atom on the β -carbon atom with an hydroxyl group (96). However, amino acids other than phenylalanine may overcome the biological action of some of these substances.

In addition to the inhibition of bacterial growth, β -3-thienylalanine and β -2-thienylalanine have been shown to retard the growth of young rats in competition with phenylalanine (400, 405).

Methionine analogs. The replacement of the sulfur atom of methionine by one of several other kinds of atoms has been found to lead to the production of agents which inhibit bacterial growth in competition with the natural amino acid. Examples of this are to be found in the replacement of the sulfur by an oxygen (giving methoxinine) (18) or by a vinyl group (25) or by one carbon atom (to give norleucine) (85, 86). Likewise, the replacement of the methyl group attached to the sulfur atom of the amino acid by an ethyl group yielded a substance (ethionine) which was toxic to rats fed a diet low in methionine (84, 403). Increase of the methionine content of the ration prevented the effects of the analog. Ethionine was the first antimetabolite of methionine to be studied and this was true both in animals and in bacteria (85). This same substance has been used to inhibit the uptake of radioactive methionine into the proteins of rats and mice, and this effect has been overcome with the metabolite (403, 421). Therefore, the conclusion has been reached that it interferes with the utilization of methionine in protein synthesis. In addition, ethionine inhibits slightly the transfer of the methyl group from methionine to choline in rats (422). However, experiments with radioactive ethionine with the label in the ethyl group have shown that rats synthesize triethylcholine from it (482, 483). Since triethylcholine mimicked the effect of ethionine on the growth of rats, and since choline overcame the action of both substances, the conclusion was reached that ethionine exerted its poisonous effect on animals by contributing to the formation of triethylcholine.

Glutamic acid analogs. Three types of structural analogs of glutamic acid have been shown to be inhibitory to the growth of bacteria, and to be reversed in this effect by increases in the amount of the metabolite in the medium. Thus, the replacement of the γ -carboxyl group with a radical such as $-\text{SO}\cdot\text{R}$, where R may be any one of

several alkyl groups, has given such antagonists. Methionine sulfoxide, the compound in which R is a methyl group, seemed to be most active (63, 64, 65). Glutamine as well as glutamic acid prevented the action of these analogs, but the effect was non-competitive in contrast to the competitive antagonism shown by glutamic acid. This situation suggested that methionine sulfoxide inhibited the transformation of glutamic acid to glutamine. This view was subsequently substantiated by the finding that methionine sulfoxide competitively inhibited the enzymic synthesis of glutamine from glutamic acid.

A second kind of analog was formed by replacing one of the hydrogen atoms on the β -carbon atom with an hydroxyl group (66). A third type of antimetabolite resulted from the introduction of an alkylated amide into the γ -carboxyl group. Such compounds inhibited the growth of glutamic acid-requiring bacteria.

Analogues of other amino acids. Most of the types of structural alteration which have been found to convert an amino acid into a substance which will compete with it in bacterial growth experiments have been illustrated by the examples already quoted. A few more instances may, however, be of interest. Thus, just as with phenylalanine or with glutamic acid, the substitution of an hydroxyl group for one hydrogen atom on the β -carbon atom of aspartic acid gives rise to an antagonist of this amino acid (25). Similarly, the optical antipode of the natural amino acid serine has been found to retard the growth of bacteria, and this effect is antagonized by alanine, which differs from it in having an hydrogen atom in place of an hydroxyl group. Glycine as well as alanine is capable of overcoming the inhibitory action of serine, and, depending on the species of bacteria, so also is pyridoxine or pantothenic acid (23). The replacement of one of the methyl groups of valine by a chlorine atom has yielded a weak inhibitor of bacterial growth, the effects of which are overcome by valine, or by certain other amino acids (25). The optical isomer of naturally occurring leucine has been shown to inhibit bacterial growth and to be reversed in this action by the metabolite (81). For a strain of *Neurospora crassa*, which required lysine in the medium, arginine, a rather close structural relative, was found to be an antagonist of this amino acid (82). The structural resemblance here lies in the loss of a methylene group and the addition of an amidine radical at the end of the chain of atoms in passing from lysine to arginine. For strains of the mold which did not require lysine, arginine was not toxic. Another naturally occurring amino acid canavanine has been shown to retard the growth of several species of bacteria and to be antagonized competitively in this action

by arginine (423). Canavanine and arginine differ only in that one methylene group of the latter is replaced by an oxygen atom in the former.

Structural analogs antagonistic to purines and pyrimidines

When the ring system of the purines is changed by trading nitrogen atoms for carbons, antagonists are frequently formed which inhibit the growth of microorganisms in competition with adenine or guanine. Benzimidazole is such an inhibitory analog (16). The triazolopyrimidines also are similarly related to the purines (18), as can be seen from

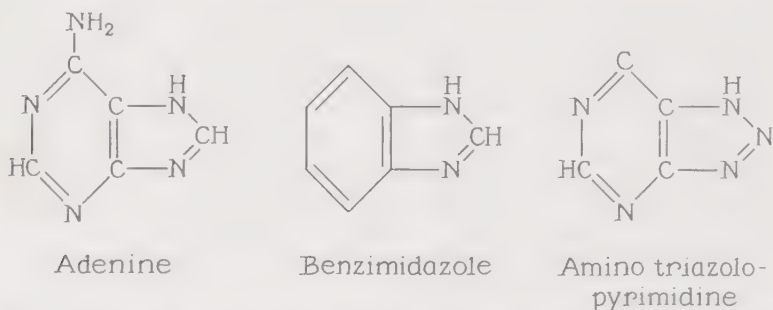


Figure 13. Structures of adenine, benzimidazole, and amino triazolopyrimidine.

the formulations of Figure 13. The effects on bacterial growth of either benzimidazole or of the substituted triazolopyrimidines may be reversed competitively with adenine or guanine, and, in the case of the triazolopyrimidines, the analogs of adenine seem to be antagonized best by adenine, and those of guanine, best by guanine. At least this situation seems to hold over a limited range of concentration. Both benzimidazole and the triazolopyrimidines are toxic to animals, but the pharmacological manifestations caused by sublethal doses are quite different for the two types of analog. Thus, benzimidazole calls forth a state resembling anesthesia (15) in which a profound lack of tone of the skeletal muscles is found, whereas the triazolopyrimidines do not produce these effects. Whereas the toxic action on several microorganisms of both kinds of analog is overcome by adenine or guanine, the effects on higher animals are neither prevented nor relieved by these purines.⁸ In addition, the ability to demonstrate antagonism between benzimidazole and adenine is not found universally with microorganisms because with one species (*Streptococcus fecalis*) it could not be observed (16).

Other kinds of alteration of the structures of purines and of pyrimidines have been made with the attendant formation of analogs which

⁸ In the unicellular animal *Tetrahymena geleii* the triazolo analog of guanine is antagonized rather specifically by guanine (484).

inhibit the growth of bacteria. A few of these also cause profound pathological changes in animals. These structural alterations consist of replacement of the groups attached to the ring system. Thus, an hydroxyl group may be exchanged for an amino, or a hydrogen atom may be replaced by an hydroxyl group or by a halogen atom. One of the most studied of such analogs is 2,6-diaminopurine, which is harmful to higher animals and to some microorganisms (19). In *Lactobacillus casei* this substance is counteracted more or less specifically by adenine. Some forms are not injured by it, and in fact it can replace guanine as a metabolite in *Tetrahymena geleii* (484). Other types of structural change of substituents on the ring system likewise succeed. Oxygen atoms may be exchanged for sulfur atoms. An example of this is seen in the bacteriocidal action of thiouracil which is overcome with the pyrimidine uracil (137). When one attempts to antagonize the toxic effects of many of the analogs of this category, however, one finds bewildering complexity. Hitchings and his collaborators (19) have made an intensive study of the inhibition of the growth of *Lactobacillus casei* by these compounds and have investigated the metabolites against which they appear to act. Some of the pyrimidine analogs, e.g., 5-nitro-uracil, seem to be antagonized in their action not by the corresponding metabolite (thymine) but rather by folic acid. Although folic acid is apparently concerned with the metabolism of purines and pyrimidines, some of the phenomena which have been described are not easy to fit into a simple concept of antagonism between structurally similar compounds. Part of the complexity probably arises from the fact that the organism has the ability to interconvert the metabolites (i.e., adenine, guanine, and hypoxanthine; or uracil and cytosine) so that the supply of a given compound available to antagonize the effects of a deleterious analog is not constant and is dependent on the functioning of a somewhat similarly constituted metabolite. This situation will be discussed at greater length in a succeeding chapter. For our present purposes it should be sufficient to note that slight alterations of the side chains attached to metabolically important purines or pyrimidines can result in the formation of toxic agents which inhibit the growth of some microorganisms, or which also may be poisonous to animals (226, 227). In many instances these harmful effects may be prevented by administration of the related metabolite, but in other instances a rather remotely similar compound may be required to do this.

Structural analogs antagonistic to histamine

A great number of compounds related in structure to histamine have been prepared and tested pharmacologically. The motive behind

these efforts has been to discover a drug for the treatment of allergies. Although the evidence is not sufficient to amount to proof, it indicates that the release of histamine is responsible for some of the manifestations of allergic phenomena. At least similar conditions can be elicited by administration of histamine. The search for an agent which would counteract the effects of histamine has therefore seemed worth while. Most of the compounds which have been studied are structural analogs of histamine, although appreciation of this fact came only after much

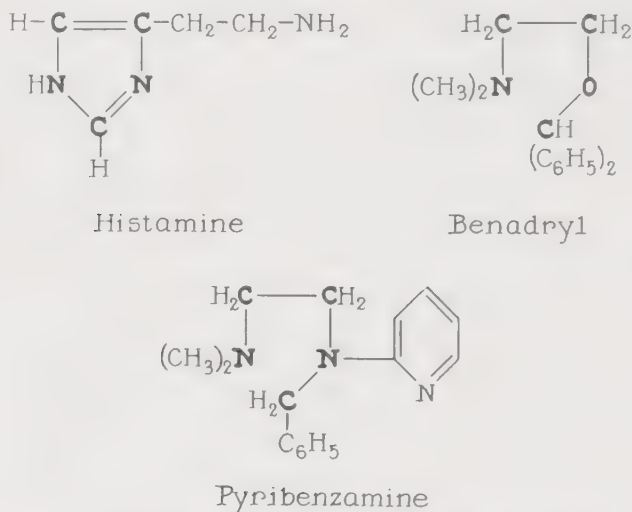


Figure 14. Structures of histamine, benadryl, and pyribenzamine. The elements in heavy type are the ones which may be considered analogous in the three compounds.

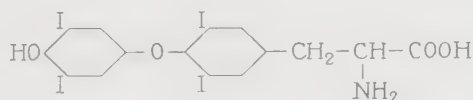
empirical testing (31, 76). However, the analogy is not as close as that between sulfanilamide and *p*-aminobenzoic acid, but does compare favorably with that between succinyl sulfathiazole and *p*-aminobenzoic acid. As the chemical nature of active antihistamine agents is modified more and more with the aim of improving their pharmacological action, the analogy to histamine structure may grow less clear. Many of the most efficacious drugs may be viewed as histamine in which the ring has been opened at the number 2 carbon atom. In addition, the nitrogen atoms thus exposed have been alkylated, and frequently one of them has been replaced by an oxygen atom which then bears a suitable substituent. Usually, the alkyl side chain of histamine has been eliminated or moved down to the adjoining nitrogen atom or its substituent. These changes may be traced by comparison of the structure of histamine with that of benadryl and pyribenzamine, two clinically useful antihistamine drugs (see Figure 14). The alkylation of the nitrogen atom may proceed so far that it is incorporated into a pyridine ring in

which the number 4 carbon atom of the original histamine has become the α -carbon atom of this same pyridine ring.

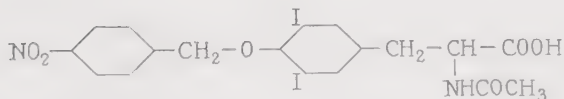
These antihistamine compounds are usually tested by determination of their ability to overcome histamine-induced shock of experimental animals. Frequently, the assay consists of noting the ability of the analog to overcome a histamine-induced spasm of a strip of isolated intestine. In many cases, one compound does not have good activity in all types of assay but may be quite potent in one and inactive in others. We shall not be concerned here with the enumeration of all the anti-histamine agents which have been produced, for their number is very large. Furthermore, several reviews dealing with them are available (68, 76, 228). However, in a subsequent chapter it will be noted how the modification of the basic structure of a histamine analog has allowed great improvement in its pharmacological effects from a therapeutic standpoint, and from these examples some ways to build desirable qualities into antimetabolites may be learned.

Antagonistic structural analogs of some hormones

Of thyroxine. A few analogs of thyroxine have been prepared by replacement of the hydroxydiiodophenyl portion of the hormone with



Thyroxine



p-Nitro benzyl ether of *N*-acetyl diiodotyrosine

Figure 15. Structures of thyroxine and of one of its antimetabolites.

p-nitrobenzyl, *p*-nitrophenylethyl, or even benzyl or butyl radicals (see Figure 15). When these compounds were tested on tadpoles, they were found to antagonize the characteristic acceleration of rate of metamorphosis and to overcome the lethal effects of small doses of thyroxine (128). Analogs made by shortening the alanine side chain of the hormone to a glycine side chain are said to be inactive as antagonists to thyroxine (229).

Of testosterone and estrone. The striking structural resemblance of the naturally occurring androgens and estrogens has been known for several years, and, coupled with their mutually antagonistic function in animals, it is indeed surprising that the view of them as antagonistic structural analogs (118) should have met with such little favor among endocrinologists. The arguments in support of this hypothesis will be discussed in a succeeding chapter, but for the present it should be noted that testosterone differs from estrone only in the state of oxida-

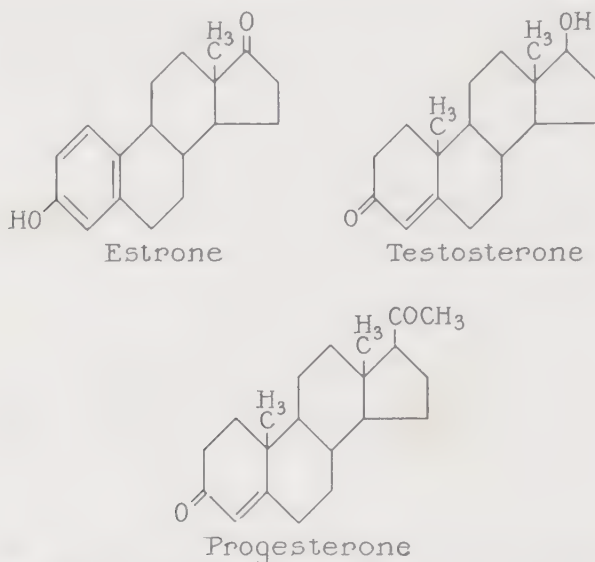


Figure 16. Structures of estrone, testosterone, and progesterone.

tion and by possession of an angular methyl group. Furthermore, progesterone differs from estrone only in the state of oxidation of the A ring, the possession of an angular methyl group in the A ring, and of a methyl ketone in the D ring (see Figure 16). Antagonism between these two pairs of hormones has been described in several endocrinological experiments (116, 117, 119). The difficulty of synthesis of steroid compounds is probably the chief reason that a variety of anti-metabolites of these hormones has not been produced in the laboratory.

Structural analogs antagonistic to intermediates in carbohydrate metabolism

A large number of examples is known of compounds analogous in structure to some of the intermediates of carbohydrate metabolism, which compete with these intermediates when they act as substrates for one of the enzymes of this process. Several of these inhibitors have been the compounds formed in the enzymic reaction, and these are, of course, related in structure to the substrate. One of the first to be

investigated was the inhibition of α -methylglucosidase by glucose (230) and of invertase by glucose or fructose, which are the products of the enzymic reaction. In many instances, however, the inhibitor is closely related in structure to the substrate but is not a product of the normal enzymic reaction. A few of these are of particular interest. They serve as illustrations of the phenomenon and must suffice, for a catalog of all the numerous individual cases cannot be presented here.

Antagonist of acetic acid. If one of the hydrogen atoms of acetic acid is replaced by a fluorine atom, the fluoroacetic acid so obtained is quite active in causing inhibition of the enzyme system of yeast which oxidizes acetic acid. The inhibition is overcome by an increase in the concentration of acetic acid provided that it is present before the inhibitor is added to the system (13). In a similar enzyme system of rabbit kidney the situation appeared to be more complex than in the yeast preparation, because the fluoroacetic acid affected more than the oxidation of acetic acid (14). In this tissue, acetic acid did not completely overcome the effects of fluoroacetic acid. Partly for this reason, all investigators have not subscribed to the idea that the action of fluoroacetic acid is due to antagonism to acetic acid. Some evidence has appeared that fluoroacetic acid actually is converted by animal tissue enzymes into fluorocitric acid, just as citric acid is believed to arise from acetic acid and oxaloacetic acid. This fluorocitric acid is viewed as the real inhibitor which then antagonizes the action of a specific enzyme which uses citric acid as substrate (424). These findings may explain adequately the action of the inhibitor in animal tissues, and it has been suggested that they may likewise clarify the observations with yeast. If this is the true explanation, then fluoroacetic acid will cease to be regarded as an antagonistic analog of acetic acid, but the exact mechanism in yeast is not yet clarified.

Antagonists of succinic acid. If the carbon chain of succinic acid is shortened by one methylene grouping, one arrives at malonic acid, which acts as a competitive inhibitor of succinic dehydrogenase (114). The normal formation of fumaric acid from succinic acid is thus prevented. This example was one of the first instances of the inhibition of an enzyme reaction by a structural analog of the substrate and is thus of historical importance. Other structural modifications of succinic acid have also proved to give rise to inhibitors of the enzyme. An example is to be found in α -sulfonosuccinic acid (115).

Antagonistic structural analogs of other metabolites

Not only with the classes of metabolites already enumerated, but also with several others, it has been found that suitable alteration of the

chemical structure leads to the formation of an agent which interferes with the normal function of the metabolite. However, there is no need to proceed further in the cataloging of these cases. We have already seen the variety of ways in which the metabolite structure may be modified, and we have also observed the array of biological systems in which the effects may be studied. Many of the examples of antimetabolites not yet described will appear in discussions of the mechanism of the phenomenon and of the applications of it which will be the concern in succeeding chapters.

CHAPTER 2

Some general aspects of the phenomenon

In the preceding chapter we have seen many examples of antagonism between metabolically important compounds and their antimetabolites. Let us now proceed to inquire whether some general principles can be deduced from this large volume of experimental observation. By so doing, existing knowledge may be clarified before proceeding to further explorations.

The functioning of one substance in a variety of biological systems

From numerous examples cited in Chapter 1 it is clear that one single antimetabolite may produce signs of deficiency of the related metabolite in higher animals, in lower animals, in fungi and bacteria or similar unicellular organisms and, finally, may exclude the metabolite from participation in an enzymic reaction in cell-free systems. This universality of action is one of the most striking general attributes of many antimetabolites and seems to arise from the ability of such agents to produce a deficiency of the metabolite. The fact that diverse kinds of living things are thus similarly affected and that inanimate enzyme systems concerned specifically with the metabolite may also be inhibited is the basis for a commonly held explanation of the mode of action of antimetabolites. This aspect of the problem will be discussed in the next chapter. However, it does not follow that any antimetabolite will affect the whole array of organisms. Many examples are known in which the analog exerts its action only in certain classes of living things. Although a given analog may affect higher animals, invertebrates, bacteria, and fungi, it usually does not harm all species of higher animals nor all kinds of bacteria. Many instances of this have been

cited in Chapter 1, and it is only necessary to mention analogs of pantothenic acid, some of which inhibit the growth of bacteria only if the latter require the vitamin to be present in the medium. Similarly, the congeners of sulfanilamide are relatively harmless to several species of mammals and birds and to certain microorganisms but yet are toxic to streptococci, pneumococci, lactobacilli, and many other types of bacteria.

Biological properties of some antimetabolites not ascribable to their antimetabolite action

Before exploring the actions of antimetabolites it is necessary to recall that all the biological effects of a compound may not be related to its antimetabolite properties, even though some of them may be. A substance may possess reactive groupings which confer on it special powers, even though the molecule resembles in structure some essential metabolite, and can be shown to act antagonistically to this metabolite in some situations. A good example of this is to be found with 2-thiouracil. This compound is the same as uracil except that the oxygen attached at position 2 has been replaced by a sulfur atom. It has been shown to function as an antagonist to this metabolite in certain bacteria (137). Not only is uracil contained within the cells of the affected species, but it is a nutritional requirement for one of them (*Staphylococcus aureus*). It is clearly of metabolic importance to them. Furthermore, thiouracil influences the germination of plant seed (404), and here too its action is overcome with uracil. However, thiouracil has an effect on higher animals which seems completely unrelated to its resemblance to this metabolite. In common with thiourea, sulfanilamide derivatives, and diverse other sulfur-containing compounds, it has an antithyroid effect. In fact, it is one of the most active of such drugs. These substances apparently function by retarding the formation of thyroxine, possibly by combining with iodide and thus removing it from the normal pathway (441). Most existing evidence is against the view that this antithyroid action has any relationship to uracil.

Competitive antagonism

Frequently, when a structural analog of a metabolite antagonizes the action of that metabolite, the two substances compete with each other. This is merely a convenient way of expressing the fact that the effect of the antimetabolite is proportional to the amount of the metabolite over a wide range of concentration of the latter. This is exactly the situation found with *p*-aminobenzoic acid and sulfanilamide, or with

thiamine and pyrithiamine (see Tables 1 and 3 of Chapter 1). Thus, if it requires 50 gammas of sulfanilamide to prevent the growth of a bacterium cultured in the presence of 0.01 gamma of *p*-aminobenzoic acid, then it will require 500 gammas of the antimetabolite to accomplish the same end in the presence of 0.1 gamma of the metabolite. When, as here, the ratio of the two antagonistic compounds is a constant over a considerable range of concentration, the relationship is said to be one of competitive antagonism, or the two substances are said to compete with each other in a particular biological function. This may be said to be the uncomplicated relationship. However, antagonism may be manifest without any evidence of competition in the sense just defined, and this state of affairs will be discussed presently.

Among enzymologists, competitive inhibition may be determined by use of the Lineweaver-Burk equation (342), which states that

$$\frac{1}{v} = \frac{1}{V_{\max.}} \left(K_m + \frac{K_m(I)}{K_I} \right) \frac{1}{a} + \frac{1}{V_{\max.}}$$

where v is the initial reaction velocity, $V_{\max.}$ the maximum reaction velocity, K_m the enzyme-substrate dissociation constant, (I) the inhibitor dissociation constant, and a the initial substrate concentration, all in moles per liter. In the case of competitive inhibition, a plot of $1/v$ against $1/a$ yields straight lines which intersect at a common ordinate intercept, provided that (I) remains constant as a is varied (343). The increase in slope is a function of (I) and $1/K_I$. This is probably a more delicate test for competitive antagonism, but in dealing with living organisms the simpler, if cruder, method indicated in the preceding paragraph is more generally used.

However, in using the less mathematical method, one must usually give heed to the initial concentration of metabolite in the system, i.e., the quantity of metabolite which the organism can synthesize. For example, in the case of the sulfanilamide-*p*-aminobenzoic acid antagonism, some of the analog must be added to inhibit growth when no metabolite has been supplied externally. This amount presumably competes with whatever *p*-aminobenzoic acid the cells can make for themselves. When metabolite is now added and the quantity of analog needed to counteract it is determined, the amount actually needed is not this quantity, but rather this amount minus that necessary in the absence of added metabolite. Obviously, this is only a factor in those organisms which synthesize the metabolite and does not apply to those which depend on external sources.

The inhibition index

In measurement of antagonism between metabolite and antimetabolite the potency of the inhibitory analog may be expressed as a number known as the inhibition index. It is the quotient of the ratio of concentration of the two substances at which their effects are just counterbalanced. It represents the amount of antimetabolite which is needed to overcome the effect of a unit weight of metabolite. In the case of sulfanilamide and *p*-aminobenzoic acid cited in the previous section, the inhibition index would be 5,000, since 5,000 times as much sulfanilamide as of *p*-aminobenzoic acid will result in inhibition of bacterial growth and less than 5,000 times as much will not.¹ To determine this index, therefore, the quantity of antagonistic analog needed to exert an effect is measured in the presence of a known amount of the metabolite, and then this determination is repeated with multiple amounts of metabolite. The relationship frequently is seen most clearly when enough metabolite is used to meet the normal requirements of the system. If less than this amount is used the inhibition index may not always be the same as that found with larger amounts of the metabolite. In other words, it is usually advisable to start with a concentration of metabolite sufficient to saturate the system before experiments are made with an analog with the object of measuring inhibition index or of determining whether the index is constant. Although it is not always necessary to do this, because with some metabolite-antimetabolite pairs the inhibition index is constant even when subnormal amounts of metabolite are present, situations in which the index changes markedly when subnormal concentrations of the metabolite are used are to be found with the quinoxaline analog of folic acid, namely quinoxaline-2-carboxyl-*p*-aminobenzoylglutamic acid, and also with some analogs of other vitamins and amino acids. Therefore, this point must be borne in mind when inhibition indices are measured.

¹ In practice, some question may arise about the selection of a proper end point for the determination of the inhibition index. This is because of the shape of a dose-response curve. Frequently, it is found that when dosage of a compound is plotted against the biological response, the curve rises regularly until the range of maximal effect is approached; then it may flatten out to an asymptote. This shape of the curve makes it impossible to decide with accuracy the dose which gives maximal effect. A much more exact estimation can be made by determination of the dose corresponding to half maximal effect. In calculating inhibition indices, therefore, it is more advisable to judge the end point at which metabolite and antimetabolite are exactly opposed to each other, by use of doses of antimetabolite eliciting half maximal responses. If the end point is taken as that of the maximal effect it may be less accurate.

The inhibition index may be calculated accurately merely by determination of the amounts of metabolite and antimetabolite added to the system. This is true for living organisms which do not synthesize the metabolite. If, however, such organisms can make their own supplies of the metabolite an inaccuracy arises from the fact that the concentration of functioning substance is the amount added to the system plus an unknown amount formed within. A suitable correction for this unknown can be made by subtraction of the quantity of antimetabolite needed for an effect when no metabolite has been added. When this is deducted from the amount of antimetabolite required in the presence of various levels of added metabolite, and then the index is calculated, a more precise value will be obtained. From time to time mathematical formulae have been published to take account of this correction, but a grasp of the underlying situation and a treatment of experimental data in accord with it will eliminate the need for such rather complex equations.

The inhibition index is constant if the antagonism is competitive, since the ratio between the contending pair of substances is constant. However, if the index is not constant but changes as the concentration of metabolite is raised, the antagonism is usually said not to be competitive. In such instances an inhibition index is of little value. The situation may be illustrated well with the structural analogs of folic acid. When pteroylaspartic acid is added to cultures of bacteria which require pteroylglutamic acid as a growth factor, multiplication is inhibited. In the case of *Lactobacillus casei*, growth is reduced to half maximum when 1.6 gammas of pteroylaspartic acid is added to a medium in which 0.001 gamma of pteroylglutamic acid (folic acid) is present (167). The inhibition index is thus 1.6 divided by 0.001 or 1,600. If now the amount of pteroylglutamic acid be raised to 0.01 gamma, then 16 gammas of the analog will be needed to cause the same inhibition of growth. The quotient of the two concentrations is still 1,600. Since the inhibition index is constant we may say that the antagonism is competitive. On the contrary, if we consider a different analog of folic acid such as 4-amino-pteroylglutamic acid, we shall find that, although 0.008 gamma is needed for half maximal inhibition in the presence of 0.003 gamma of folic acid (inhibition index 2.6), only 0.02 gamma of the analog is necessary in the presence of 0.3 gamma of the metabolite (inhibition index 0.07) (161). In other words, the index is not constant, and the antagonism is not strictly competitive in nature.

If we consider for a moment only those cases in which the inhibition index is a constant, then we see that, for any given pair of metabolite and antimetabolite, the index varies from species to species. Thus,

whereas it is 2 for pyrithiamine in *Ceratostomella*, it is 33 for *Endomyces* or 500 for *Staphylococcus* (5). The magnitude is thus characteristic of the particular biological system in which the antagonism is observed. Obviously, if the index is small, the antimetabolite is very active in that particular system; if it is large, the meaning is that the antimetabolite is relatively ineffective.

For many of the antimetabolites thus far studied, the inhibition index is quite large, and this means that relatively large amounts of analog are necessary to produce an effect. Thus, for sulfanilamide acting on hemolytic streptococci, it is about 5,000. For analogs of several of the vitamins it may be as large as 1 million. For example, for the sulfonic acid analog of oxybiotin acting against biotin in yeast, the index is 1.5 million (231). Because such minute quantities of some vitamins (e.g., biotin, *p*-aminobenzoic acid) suffice to meet all needs of an organism, it is still possible to use antimetabolites with such enormous inhibition indices. If, however, appreciable concentrations of the metabolite are necessary, an analog must possess a small index if its antimetabolite effects are to be demonstrable. For example, since bacteria of many sorts require about 50 gammas of glutamic acid per cubic centimeter of culture medium, an analog of this metabolite with an inhibition index of 1,000 would have to be present in a concentration of 5 per cent of the medium before its effect could be noted. If the inhibition index were 10,000, the antimetabolite action would be undetectable because the osmotic pressure of a solution of the required concentration (50 per cent solution) would prevent growth of the test organism.

Although the inhibition index usually is large, it need not be so. The fact that it is large merely reflects our current lack of skill in designing antimetabolites. Indeed, a few cases are known in which the index is less than 1. One such is 2,3-dichloronaphthoquinone, an antivitamin K. When this analog competes with vitamin K and causes inhibition of growth of yeast, the index is 0.1 (209). Likewise, malonate acting as an inhibitor of succinic dehydrogenase has a low inhibition index. An even better example is that of the competition of carbon monoxide with oxygen for human hemoglobin, in which the inhibition index is 0.005 (232).

Non-competitive antagonism

From the examples considered in Chapter I, it can be seen that, with many antimetabolites, the relationship is not competitive. Either the metabolite is completely unable to counteract the biological effects of the analog or is able to do so only over a limited range of concentration.

Thus, dicoumarol was seen to bear a structural resemblance to vitamin K and, what is more, to call forth in animals the characteristic signs of a deficiency of this vitamin. Furthermore, these signs could be prevented by administration of vitamin K along with the dicoumarol. However, massive doses of vitamin K (several thousand times the nutritionally required amount) are needed to counteract a minimal toxic dose of dicoumarol. In addition, as the amount of dicoumarol is increased above the minimally effective one, disproportionately larger and larger quantities of vitamin K are needed to antagonize them, until, with about twenty-five lethal doses of the drug, no amount of the vitamin which it is practical to give will counteract the toxic agent.

A more extreme example is to be found in glucoascorbic acid. This substance is undeniably analogous in structure to ascorbic acid, and, when it is fed to mice along with a suitable basal ration, a syndrome is called forth which resembles in many respects the picture of scurvy in animals susceptible to it. Despite the manifold resemblances to this disease, however, the condition is neither cured nor prevented by ascorbic acid.

Another example which should be considered in this light is that of the analog of folic acid in which the hydroxyl group found in position 4 of the vitamin has been replaced by an amino group. The resemblance in structure of this compound to folic acid is striking, indeed, since the two differ in just this one slight way. When the analog is given to mice or rats or men, a disease is elicited which bears rather close resemblance to that seen in folic acid deficiency of these same species. And yet the vitamin will neither prevent nor cure the signs called forth by the analog.² Nevertheless, as one descends the evolutionary ladder, he finds that this situation begins to change. In chicks some reversal of the effects of the drug may be achieved if large doses of the vitamin are given prior to administration of the analog (119). In some folic acid-requiring bacteria the growth-inhibiting effects of the compound are overcome by the vitamin. Indeed, with some of these low forms the relationship is practically that of competitive antagonism.

From such examples we can see that two kinds of non-competitive interference with a metabolite are discernible: (1) Those in which the structurally related metabolite has very limited powers of reversal. This fact is heralded by lack of constancy of the inhibition index and by failure to achieve any reversal of the effects of large doses of anti-metabolite. In such instances the action of small amounts of the analog

² Compare footnote 5 of Chapter 1 and also the discussion of analogs of folic acid where it was noted that some investigators have reported the overcoming of toxicity of this analog for mice by large amounts of folic acid.

may be nullified by the metabolite. (2) Those for which the structurally similar metabolite does not reverse the effects even though the attempt to do so be made at minimal doses of the harmful agent. The only involvement of the metabolite, therefore, is that the harmful agent is a structural relative of it and that the signs which the analog calls forth are similar to those seen in a deficiency of the metabolite. Probably case (2) is the ultimate extreme of (1).

The problem of interpretation of the phenomenon of antagonism between structurally similar compounds would be simplified greatly if only the examples of competitive antagonism were considered. Thus, the inhibition of bacterial growth which an analog might cause could be explained as the induction of a deficiency of the related metabolite, and as evidence for this view the precise competition of the two substances could be cited. For any amount of the inhibitor a proportional amount of the metabolite would completely eliminate its effects. The case would be even stronger when the competition could be demonstrated in animals, because here there would be the additional evidence that the analog elicited the characteristic signs of a deficiency of the metabolite. These signs are numerous, and some are rather specific. Several investigators are of the opinion that only these instances of competitive antagonism should be considered as examples of antimetabolites. If the effects cannot be antagonized by the structurally similar metabolite, these workers have maintained that the analog cannot be said to interfere with the action of the metabolite (31, 185). A survey of all existing evidence, however, leads inevitably to the conclusion that this opinion should be modified. The problem of deciding which cases shall belong to this phenomenon of the antimetabolites, and which shall be excluded, is not simply one of determining whether the antagonism is competitive. What are we to say about dicoumarol and its relatives which call forth the signs of the deficiency, but for which the related metabolite has only weak and limited powers of reversal of the action? What are we to conclude about the amino analog of folic acid for which the demonstration of competition depends on the species? Even consider the classical example of the sulfonamide drugs. Although the competitive reversal of their growth-inhibiting action by *p*-aminobenzoic acid has been shown adequately in a large number of bacterial species, a few organisms have been discovered for which *p*-aminobenzoic acid has no antisulfonamide effect, e.g., *Bacillus tularensis* (233). The dilemma with which we are faced in this matter stands out clearly when the case of phenylpantothenone is considered. This close structural analog of pantothenic acid inhibits the growth of a considerable variety of microorganisms. For those which

require pantothenic acid as a growth factor, the toxic action of the analog is overcome completely and competitively by the vitamin, but for those which make their own supplies of pantothenic acid the action of the analog is not overcome at all by the metabolite. Representative data to illustrate this point have been taken from the literature (169) and are shown in Table 1. If only the former species had been exam-

Table 1

Amounts of phenyl pantothenone required to reduce growth of various microorganisms to half maximum in presence of 0.04 γ of pantothenic acid per cubic centimeter

Organism	Phenylpanto- thenone, gammas per cc.	Reversal by Pantothenic Acid	Pantothenic Acid Requirement
<i>Lactobacillus casei</i>	28	Yes	Required
<i>Lactobacillus arabinosus</i>	180	Yes	Required
Hemolytic streptococcus, strain 0-90 (Group B)	60	Yes	Required
<i>Escherichia coli</i>	2,000 *	No	Not required
<i>Staphylococcus aureus</i>	140	Yes	Not required but slightly stimulatory
<i>Saccharomyces cerevisiae</i>	33	No	Required but replaceable by β -alanine
<i>Endomyces vernalis</i>	39	No	Not required

* Complete inhibition of growth was not obtained with this organism.

ined, there would have been little question about considering phenylpantothenone as a bona fide antimetabolite, but, if only the latter species had been encountered, what would have been the conclusion?

Some have maintained (e.g., 31, 185) that the conclusion should be that the analog acts as an antimetabolite in the species where reversal by the metabolite can be shown but is merely a poisonous substance unrelated in action to the structurally similar metabolite in the other species. In considering this view, it is instructive to note the following facts. Methylfolic acid, which is related to the vitamin in that one hydrogen atom at position 7 or 9 has been replaced by a methyl group, is a competitive antagonist to folic acid in the growth of some lactic acid bacteria. However, not only the structurally related vitamin but also the dissimilar metabolite thymidine reverses the growth-inhibiting action of this analog (372). The antagonism by thymidine is non-competitive in contrast to that of folic acid but it is clearly evident. This and other biochemical information has implicated the functioning of folic acid in the biosynthesis of thymidine as we shall see in more detail in Chapters 3 and 10. In contrast to the observations with methylfolic acid, the toxicity for *Escherichia coli* of 4-aminopteroyl-

glutamic acid is not overcome by the vitamin. This is one of the species in which antagonisms of the structural analog and the metabolite cannot be shown, and hence, if the view just propounded were followed, the action would be attributed to unknown causes not connected with the antimetabolite phenomenon. However, thymidine counteracts the effects of 4-aminopteroylglutamic acid on *Escherichia coli* (425). In addition, this analog evokes in higher animals many signs which are seen in a dietary deficiency of folic acid. Similar experiences have arisen with some of the derivatives of sulfanilamide. Thus, although the antibacterial action of sulfanilamide, sulfapyridine, or sulfadiazine may be antagonized competitively by *p*-aminobenzoic acid, that of *p*-aminobenzene sulfonyldibromoanilide cannot be. Nevertheless, this anilide appears to be as closely related structurally to the metabolite as are the other analogs just mentioned. Methionine overcomes the antibacterial action of sulfanilamide, sulfapyridine, or sulfadiazine. For this reason, and also because methionine will replace *p*-aminobenzoic acid as a growth factor for certain selected strains of bacteria (375, 426), the opinion has arisen that this amino acid is intimately related to the functioning of the other metabolite. Methionine will overcome the antibacterial action of the dibromoanilide, even though *p*-aminobenzoic acid will not (140). Similar situations have been observed with phenylpantothenone in relationship to glutamic acid in the growth of microorganisms (198), and with glucoascorbic acid in relationship to a substance in green plants in the growth and well-being of mice (26). These antagonisms by structurally dissimilar compounds will be considered in a succeeding section of this chapter. For the moment, they bear on the question of how to interpret non-competitive antagonism between structurally similar compounds.

Obviously, the reliable test for inclusion or exclusion of a case among the antimetabolites is not the question as to whether the antagonism is competitive, or even whether the related metabolite can overcome the toxic action of the analog. Without this evidence, the argument is greatly weakened, but it is not the sole criterion. To decide between those cases which belong among the antimetabolites, and those which do not, is quite similar to the problem of determining whether a given reaction is due to an enzyme. This decision also is frequently not clear. Heat lability of the active material is no certain test, because some enzymes are known which are rather resistant to high temperatures, and specific proteins (e.g., hemoglobin) are known which are destroyed by heat but are certainly not enzymes. The criterion of persistence of the enzyme in the reaction is not sufficient either, because cases are known in which the enzyme is destroyed during the reaction.

In enzymology, there are a family of properties which enzymes possess, and, although one member of the group may not have every single one of these, each does have a majority of them. The situation is similar with the antimetabolite problem. In succeeding chapters reasonable hypotheses will be explored which help in understanding some of the complex (i.e., non-competitive) relationships of these substances.

Cases in which the order of addition is crucial

Let us pause now to consider a number of examples of a situation which is by no means universally encountered, but which is found frequently enough to deserve attention. As will appear in subsequent chapters, this situation may be of importance to those who wish to devise new therapeutic agents. The situation is that the action of several antimetabolites can be overcome by the related metabolite only if that metabolite is admitted to the test system prior to the analog. For example, β -acetylpyridine will call forth in mice or in dogs many of the signs of nicotinic acid deficiency. These signs are severe enough to terminate in the death of the animal. If increased amounts of nicotinic acid have been given before the acetylpyridine is administered, these manifestations can be prevented completely. Indeed, the amount of nicotinic acid needed to protect a mouse is proportional to the amount of acetylpyridine which will subsequently be given. In other words, the antagonism is competitive. However, if the acetylpyridine is given before the administration of nicotinic acid, large doses of the vitamin are unable to prevent the lethal effects of the analog (94).³ Similarly, the toxicity in rats of some arsenical analogs of *p*-aminobenzoic acid like atoxyl (*p*-aminophenylarsonic acid) or of carbarsone (the urethane of atoxyl) may be prevented by prior dosing of the animals with the metabolite. Once poisoned with the arsenical compound, however, the animals cannot be saved with the metabolite (42). Indeed, both with β -acetylpyridine and with these arsenical drugs the corresponding metabolite must be given to the animal several hours before the analogs if antagonism is to be demonstrated. Again, desoxypyridoxine is capable of eliciting the signs of pyridoxine deficiency in rats or in chickens, provided that the intake of this vitamin has been suboptimal. If enough pyridoxine has been ingested to meet the nutritional needs of the animal, the analog is almost ineffectual (102, 146). Other examples have already been noted, such as the findings with 4-aminopteroylglutamic acid. This analog produces in a variety

³ In the section dealing with antimetabolites of nicotinic acid in Chapter I, it was pointed out that the importance of the order of addition of acetylpyridine is not found universally among biological test systems.

of animal species the signs of folic acid deficiency, and these cannot be cured by administration of this vitamin. However, in some animal forms (e.g., chicks and possibly tadpoles) some protection against the harmful effects of the aminopteroylglutamic acid can be achieved by prior treatment with large amounts of folic acid (119).

This situation can be shown not only with higher animals but also with other biological test objects. It is even found in enzyme preparations. For example, the oxidation of acetic acid by resting yeast cells is inhibited by fluoroacetic acid. The specificity of this action may be appreciated from the fact that only the fluoro compound and not the chloro or bromo analog is effective. If the metabolite (i.e., acetic acid) is added to the system before the analog, then the inhibition is erased, but, if the reverse order of addition is carried out, then no nullification of the toxic action is found (13). Whichever of the two competitors gets to the site of action first is most likely to remain there when its structural relative arrives.

It should be emphasized that this dependence of the ability to demonstrate reversal of antimetabolite action on the order of administration of metabolite and analog is not a universal finding. It is present with a rather large number of antimetabolites, but numerous cases exist in which it does not obtain. Witness, for example, the sulfonamide drugs, pyriethamine, most of the antagonists of pantothenic acid, and the antimetabolites derived from folic acid by means other than the introduction of an amino group in position 4. All these cases, and many more, could be cited to show that, with a variety of metabolite-antimetabolite pairs, the order of administration of the substances is immaterial.

Antagonism between structurally dissimilar compounds

Although this monograph is concerned primarily with the biological antagonism which exists between metabolites and their structural analogs, some attention should be drawn to antagonisms which are found among compounds quite dissimilar in chemical structure. The chief reason for mentioning this second type is that insight into the metabolic functioning of a biologically important compound has frequently been gained by knowledge of these dissimilar antagonists.

The antagonism which is found between structurally unrelated compounds is usually non-competitive. This is best illustrated by consideration of a few examples. Thus, the inhibition of bacterial growth caused by phenylpantothenone is overcome competitively by the related metabolite, pantothenic acid. However, the inhibition may also be overcome by glutamic acid or histidine, neither of which is structurally

very similar to the antivitamin. The amounts of these latter compounds which are needed to produce reversal of the effects of phenylpantothenone are large in comparison to the dose of pantothenic acid. Thus, when phenylpantothenone inhibits the growth of *Lactobacillus casei*, 2 to 3 milligrams of glutamic acid are needed to accomplish the same reversal as can be achieved with 0.04 gamma of pantothenic acid (198). Furthermore, a fivefold increase in the concentration of the growth inhibitor cannot be counterbalanced by a corresponding increase in the amount of glutamic acid present in the medium. In other words, glutamic acid will antagonize the action of phenylpantothenone when the latter is present in small amounts, but not when the dose is large.

A similar situation is found with the derivatives of sulfanilamide. Not only the structurally similar *p*-aminobenzoic acid but also the quite dissimilar methionine or adenine likewise will antagonize the antibacterial action (234). Here too it can be seen that, whereas the antagonism between sulfanilamide and *p*-aminobenzoic acid is competitive, that between sulfanilamide and methionine or adenine is not strictly so (235). The amount of methionine or of adenine required to antagonize a given dose of sulfanilamide is quite large in comparison to that of *p*-aminobenzoic acid.

If only the structural analogs antagonistic to the vitamins are considered, it is striking to note the frequency with which these dissimilar antagonists turn out to be amino acids. The cases of pantothenic acid analogs and of *p*-aminobenzoic acid analogs have already been noted. Another example is the protection of mice against the toxic manifestations of β -acetylpyridine (a nicotinic acid analog) brought about by tryptophane (236).

In many situations, as was pointed out in one of the preceding sections, the structurally dissimilar metabolite is a more universally effective antagonist to the antimetabolite than is the related metabolite itself. Thus, methionine will reverse the antibacterial properties of the dibromoanilide of sulfanilic acid, whereas *p*-aminobenzoic acid will not. The former structurally dissimilar metabolite is one of those which likewise interferes with other sulfonamides. Again, the toxicity of phenylpantothenone for microorganisms is nullified by the related metabolite (pantothenic acid) only if pantothenic acid is a nutritional requirement for the species under examination. However, the structurally dissimilar metabolite glutamic acid antagonizes the action of phenylpantothenone in species which do not require pantothenic acid as well as in those which do. In other words, glutamic acid will cause reversal in more microorganisms than will pantothenic acid. Other

examples of this phenomenon are known but these two are sufficient illustration.

It is clear from this and the preceding sections that there are two distinct types of non-competitive antagonists. (a) Those which bear structural resemblance to the metabolite, such as dicoumarol and 4-aminopteroylglutamic acid. (b) Those which are unrelated structurally (or very distantly related) to the metabolite, such as methionine and the purines with sulfanilamide, or thymidine with 4-aminopteroylglutamic acid. In the next chapter it will be seen that the basic mechanism of action of these two types is probably quite different. The first possibly function as antimetabolites which are bound to the site of action of the related metabolite; the second may be products of reactions involving the metabolite, which when they are supplied, circumvent the need for it.

Although the antagonism between structurally dissimilar compounds such as those just discussed is usually non-competitive in nature, this is not always true. One illustration of this is found with 2-chloro-*p*-aminobenzoic acid. This analog of *p*-aminobenzoic acid inhibits bacterial growth, and this action may be antagonized competitively with the related metabolite. However, the toxic effects are also overcome by pantothenic acid, and in this instance as well the antagonism is said to be competitive even though the metabolite pantothenic acid is not related structurally to the toxic agent (237). Such situations show again that the mere existence of a competitive antagonism is not an adequate criterion by means of which to limit the field of antimetabolites. However, in discussing antagonism between structurally dissimilar compounds, we are certainly at the borders of the field, if we are not beyond them.

Stimulation of living organisms with antimetabolites

During the testing of antimetabolites as inhibitors of microbial growth it is quite common to find that subinhibitory concentrations result in stimulation of growth. This stimulation may be quite pronounced, and it usually is not possible to duplicate it by giving any amounts of the related metabolite. Thus, sulfanilamide may stimulate the growth of bacteria, and *p*-aminobenzoic acid will not duplicate this effect. Similarly, the growth of some bacteria may be enhanced by small concentrations of pyriethamine, too small to cause inhibition, and this enhancement cannot be duplicated with thiamine. Likewise, benzimidazole (16) and the dichlorosulfanilide related to pimelic acid (314) have been observed to increase growth above that normally obtained. Such stimulation by antimetabolites has usually been observed

in living organisms, and not in isolated enzyme systems, and this fact may be of significance in attempting an explanation of the situation, but it will be seen in the next chapter that an interpretation of it is difficult. Meanwhile, it is well to remember that poisonous substances of all sorts are sometimes found to be stimulatory when administered in subtoxic amounts to living organisms. The antimetabolites are no exception to this.

Dependence of the activity of an antimetabolite on the nutritional requirements of the organism

Among the antimetabolites related to the vitamins, the ability to cause signs of deficiency is frequently dependent on the nutritional requirement of the organism for the analogous metabolite. If the species under investigation can make its own supply of the vitamin, the structural analog does not affect it, whereas, if it depends on an outside source of the vitamin, it is susceptible. This point was well illustrated by the data given in Table 3 of Chapter 1, in which it was shown that only those organisms which require thiamine (or its component halves) in the medium were inhibited in growth by pyrithiamine. A similar situation holds for some of the analogs of pantothenic acid such as for pantoyltaurine (177), and for pantothenol. Data to illustrate this fact with pantoyltaurine have been taken from the work of Snell (239) and are shown in Table 2. Likewise, analogs of folic acid, such as pteroyl-aspartic acid, methylfolic acid, and 7-hydroxy-9-oxypteroylglutamic acid, inhibit only those bacteria which need folic acid as a growth

Table 2

Amounts of pantoyltaurine required to inhibit growth of various bacteria correlated with nutritional requirement for pantothenic acid. Tests were conducted in the presence of 0.03 gamma of pantothenic acid per cubic centimeter.

(Inhibition index calculated for half maximal inhibition.)

Organism	Inhibition Index	Pantothenic Acid Requirement
<i>Lactobacillus arabinosus</i>	1,000	Required
<i>Lactobacillus pentosus</i>	1,000	Required
<i>Streptococcus fecalis</i> R	1,000	Required
<i>Propionibacterium pentosaceum</i>	500	Required
<i>Leuconostoc mesenteroides</i>	70,000	Required
<i>Escherichia coli</i>	No effect with 5 mg. per cc.	Not required
<i>Staphylococcus aureus</i>	No effect with 0.1 mg. per cc.	Not required
<i>Shigella paradysenteriae</i>	No effect with 0.1 mg. per cc.	Not required
<i>Brucella abortus</i>	No effect with 0.1 mg. per cc.	Not required

factor.⁴ This is well illustrated by the data in Table 3 which have been taken from the literature (166). Other examples may be found

Table 3

Inhibition of microbial growth caused by 7-hydroxy-9-oxypteroylglutamic acid (2-amino-4,7-dihydroxypteridine-6-carboxylyl-*p*-aminobenzoylglutamic acid), correlated with nutritional requirement for pteroylglutamic acid. The tests were done in the presence of 0.001 gamma of the vitamin per cubic centimeter.

Organism	Amount to Cause	
	Half Maximal Inhibition, gammas per cc.	Pteroylglutamic Acid Requirement
<i>Lactobacillus casei</i>	0.7	Required
<i>Streptococcus fecalis</i> R.	11.0	Required
<i>Lactobacillus arabinosus</i>	No effect at 50	Not required
<i>Leuconostoc mesenteroides</i>	No effect at 50	Not required
<i>Escherichia coli</i>	No effect at 50	Not required

among antimetabolites related to riboflavin, pyridoxine, biotin, and nicotinic acid.

Although the correlation of nutritional need for the metabolite with activity of the analog is very often found, it is by no means universal. Many antimetabolites are known which affect organisms without regard to their requirements for structurally related growth factors. Sulfanilamide and its derivatives are good examples, because these drugs inhibit the growth of bacteria whether or not they can synthesize their own supply of *p*-aminobenzoic acid. Similarly benzimidazole does not discriminate between species which require adenine and those which do not, and 2,3-dichloronaphthoquinone does not single out and inhibit only those which need vitamin K.

Two examples intermediate between these extremes are worthy of note. Phenylpantothenone inhibits the growth of microorganisms whether or not they require pantothenic acid. It thus differs from several other antimetabolites of pantothenic acid and would fall into the second category discussed above. Although its action is overcome by pantothenic acid in those species which require the vitamin, for those which make their own supply, pantothenic acid is unable to nullify the effects of the analog. (See Table 1.) The other example involves glucoascorbic acid. It is quoted to illustrate that these relationships which have been established almost solely with microorgan-

⁴ When sufficiently large amounts of 4-aminopteroylglutamic acid are used, certain bacteria such as *Escherichia coli*, which do not need folic acid, are also inhibited in growth. The concentration of analog, however, which is necessary is several thousand times that which suffices for folic acid-requiring species (425).

isms as test systems probably extend into the higher animals as well. This analog of ascorbic acid produces a disease in either mice or guinea pigs when it is fed along with a highly purified diet. In guinea pigs, a species which needs ascorbic acid, the effects of the analog may be largely overcome by small doses of the vitamin, but, when mice, which make their own ascorbic acid, are used, the vitamin is incapable of counteracting the effects of the drug. In Chapter I some obscurities about the action of glucoascorbic acid were described, so that a better example of the point we are discussing is desirable, but, until more elaborate experiments are done in animals with antimetabolites, this one will have to suffice to indicate a bridge from the exhaustive studies with bacteria to the limited ones with higher animals.

From the facts now at our disposal it would seem that for a large number of metabolites, the ability to affect an organism with a structurally analogous compound may depend on the nutritional need of that organism for the metabolite. For a series of different types of analog of the same metabolite, some may show this dependence on nutritional need, but the other types may not. Practically nothing is known yet about the structural alterations which decide this issue.

Ability of some antimetabolites to interfere with some, but not all, of the functions of a metabolite

Although many antimetabolites call forth the characteristic train of signs of deficiency of the related metabolite, and although the deficiency syndrome is frequently mimicked in considerable detail, this situation is not found universally. With certain antimetabolites, some, but not all, of the manifestations of deficiency are produced. This matter can be understood if several functions for the metabolite are envisioned. Then it would be possible for an antimetabolite to interfere with one or two of these specific functions, but not with all of them. The existence of more than one function for a metabolite is not conjectural, because in many cases it has been proved. For example, pyridoxal phosphate is known to participate not only as a coenzyme for the decarboxylation of tyrosine and other amino acids but also as an integral part of the transamination system in which an amino group is transferred from one amino acid to a keto acid with the attendant formation of a new amino acid. Again, glutamic acid may undergo one reaction to yield glutamine and may also proceed through other pathways to yield aspartic acid by transfer of its amino group to oxaloacetic acid. Thus for many metabolites, there is not one special function, but rather several.

Let us then consider a few structural analogs which may produce some of the signs of deficiency of the metabolite, but may fail to call forth all of them.

Some of the analogs of histamine constitute a case in point. Agents such as pyribenzamine are quite capable of protecting animals from shock which is induced by an excess of histamine, and in so doing they are antagonizing the action of an excess of this metabolite. However, another function of histamine is apparently not interfered with by the analog, and this is its role in stimulating gastric secretion. As a consequence, the antimetabolite will afford complete protection from shock when large amounts of histamine are administered, but nevertheless does not save the animal from death subsequently from gastric ulceration.

Somewhat similar behavior may be seen with glucoascorbic acid. Although this analog of ascorbic acid calls forth in mice the gingival and petechial hemorrhages, the edema of the ankles, and the decalcification of bones which are seen in scorbutic men and guinea pigs, it does not elicit the changes in tooth structure which also are characteristic of the deficiency disease.⁵

These examples have dealt with intact animals, and the effects of the antimetabolites have been judged by pathological and histological changes. This introduces many complexities in the interpretation, and it may be said that the failure of the analog to affect all functions of the metabolite is because the analog is prevented from reaching all sites of action of the metabolite. This is a plausible explanation which probably applies in many situations and may adequately account for many of the observed facts. However, it is interesting to note that similar failures of a given antimetabolite to affect all functions of a metabolite have been observed in enzyme systems. Because such systems have reduced the complexity inherent in a living organism, they are worthy of note in this connection. Pyriethamine and thiamine constitute a case in point. Although this analog of thiamine is able to compete with the vitamin and thus inhibit the synthesis of cocarboxylase, as was described in Chapter 1, it does not interfere with the vitamin when it is acting as a substrate for the thiaminase of fish tissue (238). This enzyme attacks thiamine in such a way as to remove the thiazole moiety and to transfer the pyrimidine portion to an acceptor amine (427), and this reaction is not inhibited appreciably by the analog (428, 429).

⁵ Indeed, this fact has been used to argue that the condition which glucoascorbic acid causes is not at all related to scurvy (29).

Thus it is clear that certain antimetabolites may antagonize only some, but not all, of the functions of a given metabolite. Why this should be so may possibly find some explanation in the succeeding chapters, although it is probable that these reasons are diverse and vary from case to case.

Epilogue

Some of the general features of the phenomenon of antimetabolites as they appear in 1950 have been portrayed in this chapter. As more work is done, new generalizations will probably become evident and the character of some of the old ones may be modified to a considerable extent. In 1960 some of the features which appear generally applicable now may emerge only as special cases, or even as exceptions to new generalizations.

CHAPTER 3

Hypotheses about mechanism of action of antimetabolites

Having seen briefly many of the examples of competition between structurally similar compounds, and some of the general features of the phenomenon, let us turn our attention to a consideration of the mechanism of action which appears to underlie them. So far as one can judge from the information now available there is a basic concept which will explain most of the facts about antimetabolites, and this basic concept may be formulated best as an hypothesis. This hypothesis arises from the views about the nature of enzyme action, and, therefore, let us consider first the mechanism of action of enzymes.

Mechanism of enzyme action

When an enzyme reacts with its substrate, and thus catalyzes a chemical transformation of that substrate, two separate but dependent processes are involved. The first is the combination of the enzyme with the substrate to form an enzyme-substrate complex. The second is the decomposition of this complex in such a way as to yield enzyme again, plus the transformation products of the substrate. The enzyme is thus regenerated and is able again to pass through the cycle, with the attendant change of more substrate into products. These products may be the two fragments into which the substrate has been decomposed, or they may be molecules more complicated than the substrate and derived from it by synthesis. Such a synthesis takes place by reaction of the substrate, held in an activated state in the enzyme-substrate complex, with another molecule. An example of the first situation is the decomposition of succinate to yield fumarate and two hydrogen atoms as depicted in Figure 1. An example of the second situation may be

seen in the enzymic synthesis of cozymase in which the substrate, nicotinamideribose phosphate, is so activated by combination with the specific enzyme that it will now react with adenosine triphosphate (the latter also possibly activated by combination with the same enzyme). The result of this reaction is the formation of inorganic pyrophosphate and diphosphopyridine nucleotide (cozymase), a product more complicated than the substrate from which it was formed (240). These

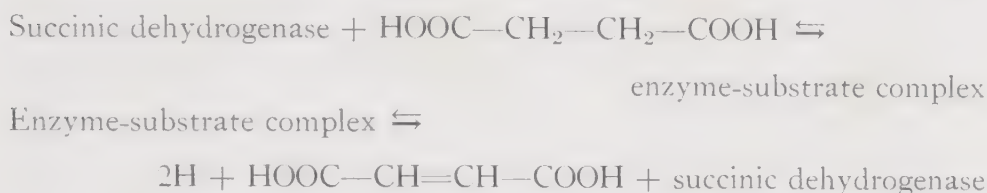


Figure 1. Enzymic degradation of succinic acid.

reactions are illustrated in Figure 2. In similar fashion sucrose is formed by the combination of glucose phosphate with a specific enzyme which so activates it that it reacts with fructose with the formation of phosphate and sucrose. Sucrose is structurally more complicated than either the fructose or the glucose phosphate. Whether the products are fragments into which the substrate molecule has been broken, or whether they are more complicated substances into which the substrate has been built, the underlying mechanism is essentially the same.

The steps in this two-stage process are reversible, so that proper alteration of the concentrations of the participants may reverse the

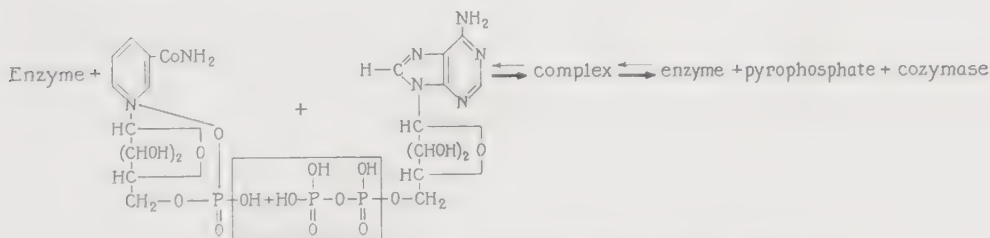


Figure 2. Reactions in the enzymic synthesis of cozymase.

direction of the overall reaction. In most cases, however, the position of equilibrium is so far to the side favoring formation of products that the reaction usually proceeds briskly in that direction. Where it is advantageous to an organism to convert the product back into the starting materials, an independent reaction involving a new enzyme and a somewhat different pathway is usually employed. Nevertheless, the processes in the two-step mechanism of enzyme action are reversible and that is the point which concerns us at the moment. Particularly we are

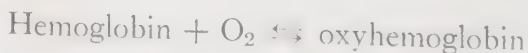
interested in the reversible combination of enzyme and substrate to form the enzyme-substrate complex, for we shall see presently that this is the point at which the action of antimetabolites is postulated to occur.

If only this first step in the reaction is considered, then it can be seen that in a mixture of enzyme and substrate, the percentage of the total substrate which will be combined with the enzyme will depend on the concentrations of enzyme and substrate relative to each other and on the specific affinity of the given enzyme for its particular substrate. This affinity is a characteristic of each enzyme and each substrate and may vary widely in magnitude from substance to substance. It is represented by the Michaelis constant of an enzyme reaction. Similarly, if the enzyme-substrate complex could be isolated, we would expect that it would dissociate into enzyme and substrate until a point of equilibrium was reached. This point would be dictated by the law of mass action. However, the enzyme-substrate complex is of such a fleeting nature (due possibly to its great reactivity) that only in a very few enzyme reactions can it be perceived experimentally; for the other cases, its existence is only postulated. Few enzymologists, however, doubt that it participates in these latter cases.

Having thus recognized the reversibility of the enzyme-substrate complex formation, and the importance of relative concentrations in establishment of the point of equilibrium, we are in a position to consider the postulated mode of action of antimetabolites. However, let us pause to discuss some semi-enzymic reactions, in which the enzyme-substrate complex is formed, but in which the second stage of the reaction, the formation of products, does not occur.

Specific non-enzymic biologic reactions which involve only combination or complex formation; "semi-enzymes"

Many reactions of metabolic importance are known in which a specific protein combines with a specific metabolite to form a complex, but in which no further reaction occurs such as is found in enzymic processes. The combinations frequently are equilibria. Because they seem to correspond to the first step of an enzymic reaction, they are of particular interest in a discussion of the mode of action of antimetabolites. The combination of hemoglobin with oxygen is such a reaction. Here a highly specific protein combines with a metabolically important substance, oxygen. We may write an equation for this reaction as follows:



The combination is reversible, and for this reason the reaction can be used by animals for the transportation of the metabolite. In a mixture of hemoglobin and oxygen, there will always be some oxyhemoglobin as well as some of the reactants. For a given concentration of the protein, the amount of free oxygen and the amount of the complex are determined by the relative concentration (i.e., pressure) of oxygen. This is particularly useful for the present discussion because the situation just mentioned has been so thoroughly investigated in relation to animal respiration. If oxyhemoglobin disintegrated to yield hemoglobin and, let us say, oxygen atoms (rather than molecules) or ozone, or hydrogen peroxide, it would be considered an enzyme-substrate complex and hemoglobin would be classed as an enzyme. However, no such products are formed, and so oxyhemoglobin is merely a complex between a specially designed protein and its specific metabolite.

Another interesting case of this kind is the combination of avidin (or antibiotin) with biotin (241, 242). The former is a highly specific protein which is found in egg white, and which reacts stoichiometrically with the metabolite biotin to yield a stable complex. The metabolite can again be recovered from this complex by destroying the protein (e.g., by heating). The combination may also be broken by altering the biotin, as by oxidation of it to the sulfone (243). The protein is then liberated largely unchanged. This case differs from that of oxygen and hemoglobin in that the combination is not reversible, and hence there is no point of equilibrium, but rather the reaction proceeds to completion in one direction.¹ In this respect it is similar to many enzymic transformations which proceed to completion and in which, enzymologists say, the point of equilibrium is far in favor of the forward rather than the reverse reaction. In the biotin-avidin combination the metabolite or the protein can be recovered only by destroying the other member of the complex. The reaction may be written in the form of an equation:

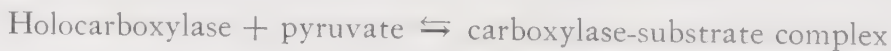


However, if this complex were to react spontaneously in such a way as to liberate avidin unchanged, and some transformation product of biotin, then avidin would surely be considered an enzyme. Since, by addition of hydrogen peroxide to the biotin-avidin complex, biotin sulfoxide or biotin sulfone is liberated, the situation approaches even

¹ The difference which arises from the reversibility of the combination is well seen when large amounts of the protein are exposed to small amounts of the metabolite. With oxygen and hemoglobin oxyhemoglobin is only partially formed, but with biotin and avidin the combination is practically complete.

more closely that of an enzymic reaction because one can envision a biochemical experiment in which, as biotin and avidin are combining, an oxidant (e.g., hydrogen peroxide) is fed into the system. The result would be an "enzymic" transformation of biotin to its sulfoxide or sulfone.

The combination of apoenzymes and coenzymes to form holoenzymes provides further examples of the matter under discussion. Here also a highly specific protein, the apoenzyme, forms a complex with the metabolite, the coenzyme. The structural requirements which must be met in the metabolite are quite exacting before combination of it with the protein can occur. The product of this combination happens to be an enzyme which participates as the catalyst in a new reaction. Thus apocarboxylase combines with thiamine pyrophosphate (or co-carboxylase). The complex so formed is an enzyme which now reacts with pyruvate as a substrate and thus catalyzes the decarboxylation of this keto acid. However, two distinct and different processes are involved: (1) The specific reaction of apocarboxylase with cocarboxylase. The product is analogous to an enzyme-substrate complex, but since the reaction goes no further the metabolite is not transformed chemically. (2) The specific reaction of the holoenzyme formed in step (1) with pyruvate to form an enzyme-substrate complex. This complex, in contrast to that of step (1) is decomposed, due to its reactivity, with the formation of the holoenzyme, carbon dioxide, and acetaldehyde. The similarity and the difference of the two reactions is thus plainly evident. The several processes may be illustrated by the following equations:



These specific combinations of protein and metabolite to yield holoenzymes have been much studied because the complexes formed play such an important role in living processes.

In connection with the question of resersibility of the reaction between specific protein and its substrate, these cases of the apoenzymes and coenzymes are illuminating. The combination of cozymase with its specific apoenzyme (i.e., dehydrogenase) is one in which the union is not firm, or in which the point of equilibrium is not far in favor of

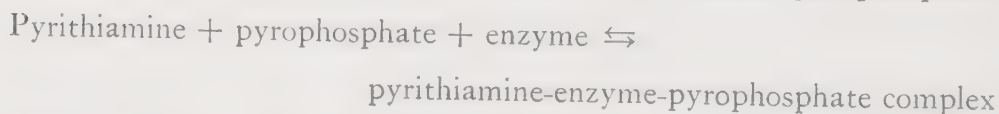
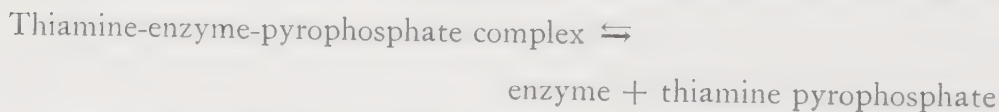
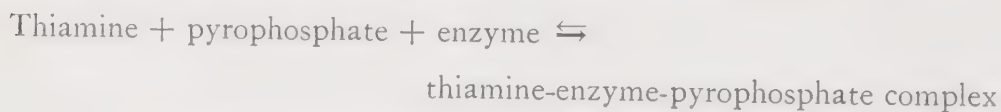
the formation of a complex. Mere dialysis will free the protein of the cozymase. We might say that this corresponds to the situation with oxyhemoglobin because a similar reduction in the concentration of the metabolite (oxygen) brings about dissociation of the complex. On the other hand, with the aspartic-glutamic transaminase, the complex which is formed between apoenzyme and its coenzyme (pyridoxal phosphate) is strong enough that the coenzyme cannot be removed by dialysis, and it is necessary to age or otherwise alter the protein part of the complex in order to secure liberation of the metabolite (pyridoxal phosphate). We might say that this is similar to the biotin-avidin complex, because here too it is necessary to harm the protein (e.g., by heating) before the metabolite can be set free. Among the holoenzymes which have been studied all degrees of ease of breaking of the combination (i.e., of liberation of the coenzyme) have been encountered.

A fourth instance of specific complex formation between a special protein and a particular "substrate" molecule should be considered. This is the reaction of an antibody with its antigen. From many standpoints this combination is best viewed as quite analogous to the formation of an enzyme-substrate complex and differs from an enzymic reaction only in lacking the second stage of the latter process. The structural specificity required in the antigen is well known as is also the uniqueness demanded of the protein or antibody component. Furthermore, some of the complexes which are formed can be made to dissociate into the original reactants by suitable alteration of physical conditions. Just as with the coenzyme plus apoenzyme reactions, the ease of reversibility of antigen-antibody combinations varies widely from case to case. The reaction can be inhibited by compounds similar in structure to the antigen, and these are known among immunologists as haptens. If this view of the antibody-antigen reaction be correct, then the antibody would appear as an incomplete enzyme elaborated by the body to meet a new challenge in the form of a foreign antigen. One might even speculate that, if this challenge were presented continuously and long enough, the antibody might be further evolved to the point at which it was an enzyme, that is, to the point of being able to carry out the second step of an enzymic process which would result in the destruction of the antigen. The antibody might thus be said to be a semi-enzyme. So also hemoglobin, avidin, and apocarboxylase would be semi-enzymes. These are, of course, speculations, but the fact of specific complex formation between the antibody and the antigen is well established.

The favored hypothesis to explain the mechanism of action of anti-metabolites

The most plausible explanation of the phenomenon of antagonism between structurally similar compounds is that the antimetabolite is able to form a complex with the enzyme, or other specific protein, with which the metabolite normally reacts, but that, unlike the enzyme-substrate complex, this enzyme-antimetabolite complex cannot then be converted into the normal products. The result is that the cyclic, two-stage process by means of which fresh enzyme is continually liberated is stopped, and the supply of products from the reaction is cut off. Like the fabled dog in the manger, the analog cannot use the enzyme, and it prevents the metabolite from doing so as well.

Consideration of a few examples will clarify this hypothesis. Thiamine is able to undergo an enzymic reaction in which the product is thiamine pyrophosphate or cocarboxylase. Pyrithiamine, because of its structural resemblance to thiamine, is able likewise to form a complex with the enzyme as illustrated in the following equations:



However, this pyrithiamine-enzyme complex is unable to undergo the second step in the normal process, and no cocarboxylase, and probably not any pyrithiamine pyrophosphate either, is formed. Instead, the enzyme is trapped in combination with the inhibitor. No thiamine gets pyrophosphorylated, and, as a result, the organism shows the signs of deficiency of cocarboxylase, i.e., of thiamine deprivation.

When it was first discovered that pyrithiamine would call forth in animals and in certain microorganisms signs characteristic of thiamine deficiency, some biochemists postulated that the analog probably acted by preventing the combination of cocarboxylase with its apoenzyme. However, direct experimental trial showed that it did not do this but rather that the point of action was to compete with the substrate thiamine in the synthetic process in which cocarboxylase was the product. It has not been determined whether this is the only reaction which is inhibited by pyrithiamine, and it may well be that other

processes utilizing the vitamin as substrate likewise are affected by the analog (471). It is clear, however, that pyrithiamine and cocarboxylase do not compete with each other for apocarboxylase (430). With another analog of thiamine, namely oxythiamine, a similar situation has been found (431). This compound inhibits the formation of cocarboxylase from thiamine by a partially purified enzyme system derived from yeast. Like pyrithiamine, oxythiamine does not interfere with the combination of cocarboxylase and the apoenzyme. However, when the analog is converted by chemical synthesis into its pyrophosphate and thus made analogous to cocarboxylase, this pyrophosphate does interfere with the combination of coenzyme with the protein.

Again, *p*-aminobenzoic acid combines with an enzyme, as yet unknown, and the complex thus formed is activated to react in such a way that the product is pteroylglutamic acid, or other folic acid-active material. It is probable that there are intermediate steps in the formation of pteroylglutamic acid, and that several enzymic reactions are involved, but at least it is well established that *p*-aminobenzoic acid acts as a substrate from which pteroylglutamic acid is finally synthesized (144, 244). Sulfanilamide and sulfathiazole behave similarly in causing inhibition of this synthesis. The data in Figure 3, reproduced from the work of Nimmo-Smith, Lascelles, and Woods (141), show the competitive character of the antagonism which sulfathiazole exerts on the synthesis of pteroylglutamic acid (*Lactobacillus casei* factor) from *p*-aminobenzoic acid by cell preparations of *Streptobacterium plantarum*. The enzyme systems involved in this process have not yet been separated from the bacterial cells. However, sulfanilamide, because of its structural similarity to the metabolite, also probably combines with the enzyme (or enzymes) but is unable to react further to yield pteroylglutamic acid. The enzyme is effectively engaged by the analog, and the organism suffers from the lack of pteroylglutamic acid. It is, therefore, not surprising to find that in growing cultures addition of this product of the inhibited enzyme system circumvents the harmful effects of the analog (245, 246). This has been done in some of the enterococci where it has been found that the growth-retarding action of sulfanilamide and some of its congeners can be overcome by pteroylglutamic acid. However, such an effect cannot be shown in several other species of bacteria. Rather than discuss the complexities of this particular example, let us wait for a few more chapters and at the present consider only the situation as it has been outlined. A multi-stage process such as the synthesis of pteroylglutamic acid from *p*-aminobenzoic acid is more complicated than a single enzymic reaction.

The ability of the analog to combine with the specific protein resides in its structural similarity to the normal substrate, i.e., the metabolite. This latter combines with the protein by virtue of numerous chemically reactive groupings, each of which is in proper spatial relationship to the others. The substrate thus fits the enzyme as intimately and as minutely as a key fits its lock. A molecule which also possesses most of

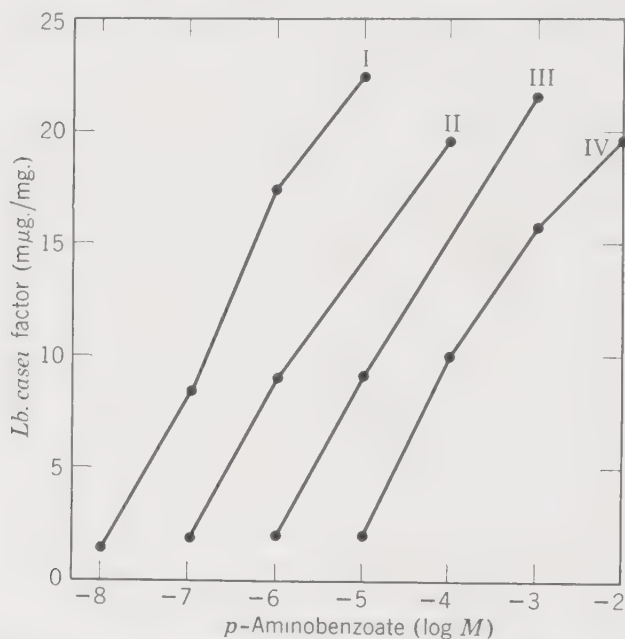


Figure 3. Inhibition of the synthesis of pteroylglutamic acid (*Lb. casei* factor) by sulfathiazole and its reversal by *p*-aminobenzoic acid. Cells incubated in simplified medium for 4 hours. Sulfathiazole concentrations: I, 10^{-7} M; II, 10^{-6} M; III, 10^{-5} M; IV, 10^{-4} M. (Reproduced with the kind permission of Dr. Woods and the *Brit. J. Exptl. Path.*)

these structural features will be able to form a complex with the enzyme, just as a key which closely resembles the proper one can be inserted into a lock even though it cannot be turned. The antimetabolite thus has the structural requirements for combination with the protein but lacks the reactive group in the proper position to allow the formation of products.

Instances are known in which the analog has not only the necessary groups for combination but also the ones for product formation as well. Some of the less discriminating enzymes may use such an analog in place of the metabolite. A foreign product is formed, but it may serve well in place of the normal one. In such a case, the analog is no antimetabolite, but rather a structural relative possessing metabolite activity. An example is arsenocholine, the analog of choline in which the nitrogen atom has been replaced by an arsenic atom. This com-

pound can combine with the enzymes concerned with the synthesis of choline into lecithin and can react as does the metabolite to form an arsenolecithin which the animal is apparently able to use in place of the normal product (145). Similarly, the triethyl analog of choline may pass through this phosphatide-synthesizing enzyme system without causing inhibition. The requirements for specificity of an enzyme apparently are not constant, for some enzymes react only with a single substance to form only one product, whereas others do not discriminate among a family of allied substances. The lecithin-forming systems belong to the latter group; the cocarboxylase-synthesizing system is an example of the former.

The formation of the enzyme-substrate complex is a reversible reaction, and so also is the formation of the enzyme-analog complex. Consideration of this situation will show why a relationship between a metabolite and an antimetabolite is usually competitive in nature. In a mixture of enzyme, metabolite, and antimetabolite, there are two competing reactions. One is for the formation of the complex with the metabolite or substrate, and the other is for the formation of the complex with the analog. The predominating reaction will depend on (1) the relative affinity of metabolite and antimetabolite for the protein and (2) the relative concentration of the two contenders. If the enzyme-metabolite complex predominates because of high relative concentration of the metabolite, and the concentration of antimetabolite is raised, a point will be reached at which the enzyme-antimetabolite complex predominates and the metabolite will be pushed off from the protein, and its place will be taken by the antimetabolite. If now the relative concentration of the metabolite is increased, the antimetabolite will be displaced from combination, and enzyme-metabolite complex will predominate. When this occurs in a living organism it can be said that the effect of the antimetabolite has been reversed, or that the metabolite has overcome the condition created by the antimetabolite.

The displacement of the metabolite by the analog is not just conjectural because in some instances it can be demonstrated in isolated systems. For this purpose semi-enzymic systems mentioned in the previous section are admirably suited because any complications which might arise due to the second stage in an enzymic reaction are automatically eliminated. Take the case of biotin and avidin. In a simplified system containing only these two reactants, combination occurs. If now biotin sulfone be added to the system in which all biotin is combined with the protein, free biotin will appear in solution (51), and some of the analog will become attached to the protein. Interestingly enough, the analogs of biotin which will thus release or displace the

metabolite from combination with the specific protein are exactly the ones which compete with biotin acting as a growth factor for micro-organisms (21).

The competition between oxygen and carbon monoxide for hemoglobin

The well-known competition between oxygen and carbon monoxide for the combination with hemoglobin may be viewed as one of the best understood and simplest examples of the antimetabolite phenomenon. Because so much information about this system is available, and more particularly because of the simplicity of the demonstration of the reactions involved, it is worth consideration. All the pertinent information was accumulated before the idea of the functioning of antimetabolites was promulgated, and only recently was the recognition of the relationship to this phenomenon advanced (118, 248).

The facts in this case are these. Both oxygen and carbon monoxide combine with hemoglobin, and with both substances the combination is reversible. When oxygen unites with the protein the extent of the combination depends on the partial pressure of this gas in the system. If this partial pressure is raised, in other words, if the concentration of oxygen is increased, then more and more of the hemoglobin will react to form the combination known as oxyhemoglobin. Conversely, if the concentration of oxygen (i.e., its partial pressure) is decreased in a system containing oxyhemoglobin, the combination breaks down with the liberation of oxygen and the accumulation of hemoglobin. These relationships are expressed in the familiar sigmoid curve obtained by plotting oxygen concentration against per cent saturation of hemoglobin. Likewise, carbon monoxide will unite with hemoglobin to form a complex, the stability of which depends on the partial pressure of the gas in the system. If carbon monoxide is introduced slowly into a system containing oxygen and hemoglobin, then, as the concentration of it rises, oxygen is displaced from its union with hemoglobin, and carbon monoxide takes its place. If, on the other hand, oxygen is admitted to a system in which carbonmonoxy-hemoglobin is present, the former gas displaces the latter, and oxyhemoglobin is formed, while carbon monoxide appears as the free substance.

The affinity of carbon monoxide for hemoglobin is greater than is that of oxygen. The ratio of these two affinities varies for the proteins from different species of animals (252), but for human hemoglobin it is about 200 (232). In other words, oxygen will not displace carbon monoxide from combination with this protein until it is present in concentration 200 times greater than that of the carbon monoxide.

These facts may be fitted to the general concept of the action of anti-metabolites in the following manner. There can be little doubt that oxygen is a metabolite, i.e., a metabolically essential substance, and that hemoglobin is a specialized protein designed to combine with it. Carbon monoxide is a substance derived from this metabolite by exchange of one oxygen atom for a carbon atom. This is one type of structural alteration which has been found effective in converting other metabolites into antagonistic agents. In the antagonism between the metabolite oxygen and its antimetabolite carbon monoxide, the relationship is competitive in nature, and, for human hemoglobin, the inhibition index is $\frac{1}{200}$ or 0.005.

This case is particularly useful for the understanding of the mechanism of action of antimetabolites because, unlike enzymic reactions, the metabolite-protein complex does not proceed to a second stage in which a product is formed and the protein is regenerated. A further advantage is that the processes can be studied in a system involving only three substances of high purity (plus, of course, water as a solvent). The actual displacement of the metabolite by the analog, and its variation with changes in relative concentration, can be measured directly. A somewhat analogous situation holds for the simplified system involving avidin, biotin, and antagonistic structural analogs of biotin, as we have seen in a previous section.

In the light of hypotheses about the atomic structure of iron and of oxygen and carbon, and about the electron shifts and interactions, a rather detailed picture may be built to explain the actual mode of linkage of protein and metabolite. (See for example (249).) On these same bases reasons can be given for the affinities of the analog (i.e., carbon monoxide) for the protein. For a detailed examination into the nature of the union of a protein and its specific complex-former, such hypotheses about atomic structure must be considered. The nature of the combining groups of each class of enzymes and other specific proteins is probably different, so that any detailed exploration of this problem would lead us too far from the subject at hand. When more is known with certainty about the nature of combining groups of such proteins, the application to studies with antimetabolites would seem evident.

Modifications of the basic hypothesis; the rate question. Toxic products

Present evidence suggests that all cases cannot be adequately explained by the hypothesis just outlined, but that special situations exist

which require some modifications of it. In this and the succeeding section some of these will be examined.

Although casual inspection may indicate that a given metabolic reaction either does or does not occur in a particular organism, more detailed study often reveals that what had been taken for a qualitative difference is merely a quantitative variation, or a difference in rate of the reaction. For example, *Lactobacillus casei* will not grow in a medium free of riboflavin but does so when this vitamin is added. *Lactobacillus arabinosus*, on the other hand, multiplies equally well in the absence of this metabolite and can be shown to synthesize it. For these reasons the accepted conclusion has been that *L. casei* lacks the ability to carry out the reactions leading to formation of riboflavin and is thus qualitatively different from *L. arabinosus*. However, when one portion of the riboflavin molecule, namely 1-amino-2-ribitylamino-4,5-dimethylbenzene, is supplied to *L. casei* in large amounts, it grows. The fragment of the vitamin is far less active than riboflavin itself, but, nevertheless, when enough of the former is present, the organism can perform the synthesis (452). Therefore, *L. casei* differs from *L. arabinosus* not in a total lack of this metabolic function, but rather in that it cannot carry the process forward at a sufficiently fast rate to allow growth to occur. If large amounts of the probable precursor of the vitamin are forced into the system, the rate can be increased to the point where growth can begin. Several other examples of this same sort of thing are being found. Thus, *Leuconostoc citrovorum* usually cannot form the so-called citrovorum factor (probably N-formyltetrahydropteroylglutamic acid) fast enough to permit growth, but, if large amounts of the biological precursor of this substance, namely pteroylglutamic acid, are supplied, growth takes place (485). Similarly, pantothenylaminoethyl sulfide is necessary for multiplication of *Lactobacillus bulgaricus*, but large quantities of pantothenic acid, its precursor, can replace it (486). In these situations, what appeared to be a qualitative failure to conduct a synthesis turns out to be only a quantitative difficulty with rate.

In the action of several antimetabolites, the real issue may be a slowing of rate of a reaction to a point where growth or other function does not occur, rather than the complete stoppage of this reaction. Some evidence in favor of such a view exists, although it is incomplete. When the triazolopyrimidine analog of guanine (8-azo-guanine) is injected into mice in sublethal amounts, the nucleic acids isolated from the viscera contain a small amount of the analog, which presumably is taking the place of a small portion of the guanine which normally occurs in these nucleic acids (487). Only about 1 of every 1,700 guanine

molecules is so replaced. The evidence thus suggests that a small part of the analog passed through reactions normally concerned with the metabolite.

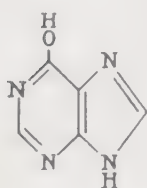
Such results have engendered the belief that the altered product, and not the administered analog, is the real harmful agent. There is no evidence that this is so, but neither is there much reason for thinking that it is not. If this is true, then the foreign product must have extraordinarily great potency, since so little of it is formed. Even if it is the real poison, a mechanism of its action must be envisioned, and this usually resolves itself into the belief that it is an analog of some nucleic acid which interferes with the biological effect of the normal one much as has been pictured for the mechanism of action of other antimetabolites. In some instances, as will be discussed in the next section, the administered analog clearly is converted into another substance before exerting its effect. In other situations, such as the one of the guanine analog, it is not yet clear whether the small amount of conversion represents a side reaction or the real path of the biological effect.

Some other kinds of circumstantial evidence speak against the formation of foreign products as the universal mechanism of action of antimetabolites. Consider the effects of salicylic acid and of salicylyl β -alanine. The former is somewhat related in structure to pantoic acid (α,γ -dihydroxy- β,β -dimethylbutyric acid). Unless the concentration is excessive, it inhibits the growth of those bacterial species which can synthesize pantothenic acid, and this effect can be antagonized with pantoic acid (247, 488). If this action were due to the formation of a foreign product, namely salicylyl β -alanine, instead of the normal one (i.e., pantothenic acid, or pantoyl β -alanine), then this substance should likewise be toxic to the organisms. However, synthetic salicylyl β -alanine is known to inhibit the growth of forms which cannot synthesize pantothenic acid, but usually not those which can (195, 196). Salicylic acid, on the contrary, shows the reverse spectrum of activity. It, therefore, seems likely that the toxic properties of the latter are not due to conversion into the former. Evidence of this sort is, of course, not entirely satisfying, and it does not preclude the possibility that a small amount of the foreign product is formed.

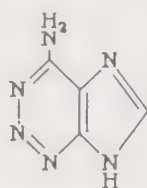
It is also conceivable in certain cases that an enzyme may react with an analog of its substrate and complete the formation of a foreign product; but this latter may not be unduly harmful to a living cell in which the enzyme is found. If the amount of enzyme is limited in relation to the quantity of substrate, then the analog, by reacting with and thus occupying some of it, may retard the normal metabolic reaction with the proper substrate. Such situations have been studied

several times with purified enzymes and have been spoken of, among enzymologists, as competitions between two substrates. The net result is an inhibition of normal product formation and superficially resembles the end result pictured for the unmodified mechanism of the preceding section. There is not sufficient evidence yet to decide whether in most cases the effects of antimetabolites are to be attributed to this sort of behavior (which is an effect on rate) or to the more extreme blocking which has been discussed in the preceding sections.

However, one antimetabolite which appears to function in this manner is noteworthy. This compound is 2-aza-adenine. When the



Hypoxanthine



Aza-adenine

Figure 4. Structural relationship of hypoxanthine and 2-aza-adenine.

carbon atom in position 2 of the purine ring system of hypoxanthine is replaced by a nitrogen atom, and the hydroxyl side chain is exchanged for an amino group, 2-aza-adenine is formed (see Figure 4). This analog is a very powerful inhibitor of the growth of several species of bacteria and fungi (499) and its action in this regard is overcome specifically and competi-

tively by hypoxanthine. Adenine is a non-competitive antagonist in that when it is present in the culture medium, even in small amounts, the aza-adenine is harmless. Xanthine shows no ability to antagonize the action of the analog. In higher animals, as well as in these microorganisms, the analog shows a toxicity (judged by death of the subjects) which may be overcome by administration of hypoxanthine, but not by xanthine. Besides these effects on living things, 2-aza-adenine will inhibit *in vitro* the enzymic oxidation of hypoxanthine to uric acid (500). That is to say, it interferes with the action of highly purified preparations of xanthine oxidase. This enzyme, which appears to function in some phases of purine metabolism of organisms, attacks either hypoxanthine or xanthine and yields uric acid. These reactions can be followed minutely, and the disappearance of substrate and the formation of product can be measured at intervals during the oxidation by taking advantage of the characteristic differences in the ultra-violet absorption spectra of each of the substances concerned.

When only analog and enzyme are present in the solution, the former is oxidized, just as is the normal substrate (hypoxanthine or xanthine) to yield the 8-oxy derivative (500). The analog is attacked as rapidly as the normal substrate. This is the situation when the enzyme is

exposed to either substance alone. When analog and hypoxanthine are mixed, however, the enzyme prefers the analog to the exclusion of the normal substrate, and consequently the oxidation of hypoxanthine is inhibited. The rate of formation of uric acid from the hypoxanthine is reduced about fivefold when the two structural relatives are present in equimolecular proportions. By noting spectroscopically the changes which take place as the reaction proceeds it can be seen that no hypoxanthine at all is attacked by the enzyme until about 80 per cent of the 2-aza-adenine has been transformed to its 8-oxy derivative. After this, the normal substrate is attacked, and at a rate only slightly below that found when no foreign substance is present in the system. When xanthine is used in place of hypoxanthine, 2-aza-adenine does not so greatly inhibit the action of the enzyme, because here the mixture of purine and analog is handled approximately as is an equivalent amount of either substance alone. Thus, just as in living things, 2-aza-adenine interferes with the metabolism of hypoxanthine much more than with that of xanthine. This does not, of course, mean that the toxic properties of 2-aza-adenine in living organisms are due only to an inhibition of the oxidation of hypoxanthine to xanthine by xanthine oxidase, but it may suggest that this is part of the mechanism.

The experimental facts show clearly in this case that the analog inhibits the action of the isolated enzyme, and that it does so by engaging this enzyme preferentially. However, the inhibitor is attacked by the enzyme, rather than merely forming a complex, and a foreign product is formed. Once the inhibitor is thus destroyed, the normal metabolic reaction then proceeds. The net result is that the normal process is slowed but not permanently stopped. In this connection, it is interesting to note that when 2-aza-adenine affects the growth of microorganisms the effect is primarily on rate of growth. If incubation is continued for sufficient time, the inhibition is no longer seen.

Clearly, the action of some antimetabolites is to exclude the normal substrate from combination with the enzyme. Malonic acid, for example, when it competes with succinic acid, cannot be dehydrogenated by succinic dehydrogenase, and it probably functions by such exclusion. The same exclusion also is at work in the case of pyriithiamine (471), because actual measurement has shown that a foreign product (pyriithiamine pyrophosphate) is not formed in detectable amounts. In other cases, however, the complicating factors of foreign-product formation, and slowing of rate of the normal process which attends it, may be at work, and may necessitate a modified explanation of the mechanism.

Transformations of analogs by living cells into the true antimetabolites

Pursuing the idea of a foreign product, we can see without equivocation that sometimes a structural analog of some metabolite may not be the actual antagonistic substance but may be converted into it by the test organisms. Perhaps the best-known example of this is to be found with prontosil, the original sulfonamide drug. This substance is the azo dye formed by azo union at the amino group of sulfanilamide. In the organism this is reduced with the consequent formation of sulfanilamide, the truly active antimetabolite (250). Another interesting example is that of desoxypyridoxine. According to current investigations (146), this substance is not active *per se* but rather is phosphorylated to yield desoxypyridoxine phosphate which now strongly inhibits the combination of pyridoxal phosphate, the coenzyme of tyrosine decarboxylase, with its specific apoenzyme. In this way the decarboxylase system is inhibited. The desoxypyridoxine itself does not seem to antagonize the phosphorylation of pyridoxal, but rather the products of phosphorylation of both substances are the real antagonists.

A third example deals with ethionine, an analog of methionine. As is well known, the methyl group of methionine is transferred metabolically and appears in choline and creatinine formed in the animal body. Ethionine, because of its structural resemblance to methionine was postulated to interfere in the utilization of this metabolite, possibly in protein synthesis, and also possibly in transmethylation. However, experiments with radioactive ethionine labeled in the ethyl group showed that this radical was transferred just as was the methyl of methionine and appeared in the choline and creatinine, presumably as the ethyl analogs of these substances (432, 482, 483). It was then shown that triethylcholine would duplicate the growth-inhibiting action of ethionine on rats, and that either methionine or choline would counteract it. Since either of these metabolites will antagonize the effects of ethionine, the real antimetabolite could be triethylcholine into which the administered ethionine was transformed. The antagonism which methionine showed to ethionine was probably due to prior conversion of this metabolite into choline. The possibility still remains that ethionine *per se* is an antimetabolite of methionine in reactions such as protein synthesis, but direct evidence of this has not yet been found.

Most of these instances may be understood when one notes that the reactions which transform the administered compound into the func-

tioning antimetabolite are general ones, rather than the highly selective type with which the antimetabolites are frequently concerned. Thus, phosphorylations and reductions are processes which the organism usually can accomplish with a host of compounds, and, although some of the individual enzyme systems show high selectivity with regard to substrate, others apparently are not so fastidious. On the other hand, there are very few compounds which can act as substrate to combine with the apoenzyme and thus, for example, to form tyrosine decarboxylase. Indeed, the remarkable structural specificity required for vitamin action is a reflection of such a fact. Only one or, at most, a very few substances possess the biological activity of a given vitamin. Therefore, it may not seem too surprising that a compound such as prontosil may pass readily through an enzymic process of reduction, and yet that its transformation product sulfanilamide should block a more specific enzymic process.

A return to the lock-and-key analogy of enzyme action may be useful in the visualization of this situation. Thus, the less important outer doors of a house frequently are equipped with rather crude locks which require only a skeleton key to turn them. A host of more or less similar keys work these locks with but slight if any inhibition. For the more vital doors, however, such as those to the strong room or to the safe-deposit vault, more elaborate locks are usually applied, and these require a rather more specific key to turn them.

The finding that ethionine is transformed to triethylcholine, and that this in turn is built into lecithin makes it possible to put forth a mechanism of action for such antimetabolites which is concerned primarily with rates of reactions and does not involve a single point of attack. Thus, it might be argued that ethionine retards the rate of transmethylation from methionine without seriously impeding this metabolic process, that the triethylcholine formed from ethionine similarly inhibits the synthesis of lecithin from choline, but not in a critical fashion, and that the triethyllecithin so formed functions as normal lecithin, but not quite so well. The action of the antimetabolite on living things would thus not be due to a simple, direct effect but rather to an accumulation of slight hindrances exerted along a chain of connected metabolic reactions. Future evidence may substantiate for many antimetabolites such a complex, diffuse mechanism, but until it does the more direct picture of exclusion of metabolite may serve to make understandable the ways in which these substances can, and sometimes do, function.

Explanation of the existing data with the favored hypothesis

The question which arises first is how well does the hypothesis about mechanism of action of antimetabolites explain the facts which are known about them. Let us, therefore, examine the existing data and attempt to determine whether the hypothesis covers the facts adequately.

Competitive antagonism. As pictured in an earlier section of this chapter, the competition (as defined in Chapter 2) between metabolite and antimetabolite is a consequence of the reversibility of the reaction between enzyme and substrate (or other specific protein and the metabolite), and between enzyme and antimetabolite. The deciding factor in determining which of the pair of structurally similar compounds actually combines with the protein in a given system is the relative concentrations of the two. In the highly simplified systems, such as those involving hemoglobin or avidin, the displacement of the metabolite when the relative concentration of antimetabolite is raised can be clearly demonstrated by direct measurement of the metabolite set free. In the more complex systems it is not so easy to show by direct measurement that this displacement takes place, but it is presumed to occur. This presumption is not wholly without experimental backing. For example, the following facts have been observed in rats given oxythiamine (251). If the intake of thiamine is constant, the excretion of the vitamin also is constant, but rises if the intake increases. If oxythiamine is now administered, the excretion of thiamine promptly rises concomitant with the appearance of signs of athiaminosis. The vitamin which is displaced by the analog is lost in the urine. Similarly, administered metabolite has been found to displace a structural analog already contained in the tissues. Thus, radioactive 3,3'-methylenebis-(4-hydroxycoumarin) is fixed for several days in the liver after it has been injected into a mouse or a rabbit. When vitamin K is given, this fixed analog disappears from the tissue (468).

The inhibition index. According to the favored hypothesis, the inhibition index would be viewed as a measure of the relative affinities of metabolite and antimetabolite for the particular proteins with which the metabolite normally reacts. For simple enzyme systems, or those involving merely complex formation as in the case of oxygen and hemoglobin, this would seem to be the only factor concerned. In living organisms, however, other factors probably must be considered. Thus the penetration of metabolite and of antimetabolite into the cell may not be equal, or the cell may possess the ability to destroy or alter the structure of the antimetabolite. Moreover, the metabolite may react

with more than one protein in the cell, and in these several processes the antimetabolite may not be equally effective. As a consequence the inhibition index as found in a living organism may represent the resultant of several forces rather than the relative affinity of the two compounds for a specific protein.

The variation of the inhibition index from species to species for the same metabolite-antimetabolite pair becomes understandable not only from what has just been said but also from reflection on the fact that, for a given substrate, the affinity for the enzyme is not the same for enzymes derived from various species. This is most clearly seen with the hemoglobins (252), or with the heme-containing oxidative enzymes. The relative affinities for oxygen and for carbon monoxide are not the same for all species. Hemoglobins from different mammals may vary in this respect by a factor of four, whereas the hemoglobin from *Gastrophilus*, an invertebrate parasite, may differ from human hemoglobin in this respect by a very large factor (i.e., 300) (252). The chemical and immunological differences of hemoglobins are well known, so that we may conclude that the affinity of protein for substrate is influenced markedly by these relatively small differences in the structure of the protein.

Non-competitive antagonism. According to the favored hypothesis non-competitive antagonism could result if the antimetabolite possessed some structural features which made the reaction of it with the enzyme not completely reversible. Some substituent of the analog may be of such reactivity that it can undergo an ordinary chemical reaction involving primary valence forces, and this may be either with some portion of the specific protein or with some other essential compound not directly concerned in the system. For example, 2,3-dichloronaphthoquinone, a close structural analog of vitamin K, will inhibit the growth of yeast and other fungi. This effect may be completely overcome by small amounts of the vitamin. However, as the concentrations of the dichloro analog are raised, a point is soon reached beyond which no antagonism can be demonstrated. At this point, the amount of vitamin which would be expected to overcome the action of the analog (on the basis of findings with smaller concentrations of the latter) is great enough by itself to inhibit the growth of the organism. Both the analog and the vitamin are quinones of a type which might normally be expected to react with free amino or thiol groups. As the concentrations are increased, it would, therefore, not be surprising to find that a generally toxic effect of either the analog or the metabolite might be encountered. It is even possible that this would begin to operate at a lower concentration of the analog than of the vitamin. Since the

analog contains chemically reactive halogen atoms it may first combine with the specific protein by virtue of its structural resemblance to vitamin K, but if enough of it is present it may be bound irreversibly by means of these reactive atoms.

While the combination of metabolite and specific enzyme may be readily reversible, that of antimetabolite and enzyme may be less readily so. It may even be completely irreversible. If the latter situation obtained, one would see the extreme of non-competitive antagonism discussed in Chapter 2, namely, that the analog calls forth the signs of deficiency of the metabolite but that these are not overcome at all by the related metabolite. In some species it may be possible to antagonize the action of an antimetabolite by the related metabolite; in others this may not be so. Several situations of this sort, e.g., with 4-aminopteroylglutamic acid and pteroylglutamic acid, have already been noted. These may result from small differences in the strength of the bond between analog and protein, depending on the species from which the protein arises. We have just seen how the hemoglobins of various organisms differ in regard to affinity for oxygen and for carbon monoxide. Consideration of these factors enables one to envision logically most of the various shades of antagonism, and to do so without leaning too heavily upon imagination.

If this is the correct view of the situation, then the reason is understandable why the order of addition of analog and metabolite to the test system is sometimes crucial in deciding whether or not antagonism can be demonstrated. In Chapter 2, several instances were recorded to indicate that antagonism could be shown only if the metabolite was admitted first. Presumably, when the specific protein is saturated with the normal substrate, and when the channels of transport of this substrate to the site of action in the cell are also completely filled, the antimetabolite must compete with this excess for combination with the enzyme. When, however, the analog can fix itself firmly and irreversibly to the enzyme, it cannot be readily dislodged by subsequent increases in the concentration of metabolite.²

In a living organism the explanation of non-competitive antagonism may have to be somewhat more involved than for isolated enzyme sys-

² Another explanation for the importance of the order of addition of analog and metabolite deserves consideration. This is that the metabolite must be transformed into a new substance before the antagonism occurs. Thus, the effect of 4-aminopteroylglutamic acid in mice can be overcome by large doses of pteroylglutamic acid provided that the metabolite is given sometime prior to the analog. Subsequently, it was found that pteroylglutamic acid is converted by mammals into a substance known as citrovorum factor or folinic acid (N-formyltetrahydropteroylglutamic acid or an

tems. Factors of penetration to the site of action and of fixation by other molecules in the cell structure adjacent to this site may be important.

There is insufficient experimental evidence with which to decide whether these ideas about the causation of non-competitive antagonism are the true explanation. It is also not clear whether irreversible chemical reactivity of the type just pictured may be only one factor involved and that others are concerned in other situations. In view of limited exact information, these points must remain speculations.

Antagonism of some, but not all, of the functions of a metabolite by a structural relative. This situation which was described at the end of Chapter 2 is understandable in relation to the favored hypothesis. Thus, although several proteins may exist, each with the ability to combine with a given metabolite, but each designed to catalyze a different type of transformation of the latter, all these proteins may not react or form complexes with the antimetabolite. The antimetabolite, therefore, would antagonize only those functions of the metabolite which are carried forward by the proteins with which this analog can form a union. No interference with the other processes in which the metabolite functions would be expected if this were true.

As was pointed out in Chapter 2, this may not be the sole explanation of the observed phenomenon. Several cases may be due to failure of penetration of the antimetabolite to each and every site of reaction of the metabolite, or to other poorly understood reasons.

Antagonism between structurally dissimilar compounds. Since certain cases of antagonism between structurally dissimilar compounds seem to lie close to the field of the antimetabolites (as was indicated in Chapter 2), let us see how they fit into the explanations arising from the favored hypothesis. First, let us consider those situations in which the dissimilar antagonist is quite obviously a product of the normal enzyme reaction in which the structurally related metabolite participates. The bacteriostatic action of sulfadiazine can be antagonized competitively by the metabolite *p*-aminobenzoic acid. It may also occasionally be antagonized by pteroylglutamic acid, a molecule much more complex than sulfadiazine, and which bears on the whole little resemblance of this); see (479). This latter material is capable of antagonizing in mice the harmful effects of 4-aminopteroylglutamic acid. Presumably, therefore, administered pteroylglutamic acid must first be converted into citrovorum factor before it antagonizes the action of the analog, and it is for this reason that the metabolite must be given before the analog if antagonism is to be demonstrated. In liver slices, and therefore probably in the intact animal, the analog prevents the formation of citrovorum factor from the metabolite (437). A somewhat similar explanation may be the correct one for other instances where prior addition of the metabolite is essential.

semblance to it. The relationship between sulfadiazine and *p*-aminobenzoic acid is competitive, but that between it and pteroylglutamic acid is not. An amount of this last substance which is sufficient to meet the needs of related bacteria for growth is sufficient to overcome completely the bacteriostasis of one effective dose of sulfadiazine, and this same amount of pteroylglutamic acid is equally effective against several hundred times this amount of the analog. These facts can be seen readily from the data in Table 1, which have been taken from the

Table 1

Amounts of *p*-aminobenzoic acid and of pteroylglutamic acid required to produce half maximal growth of *Streptococcus fecalis* (Ralston) in the presence of varying concentrations of sulfadiazine

Sulfadiazine, gammas per cc.	<i>p</i> -Aminobenzoic acid, gammas per cc.	Pteroylglutamic acid, gammas per cc.
1	0.003	0.0003
10	0.03	0.0003
100	0.3	
1,000	3.0	0.0003

work of Lampen and Jones (245). From several studies with bacteria (141, 244) it is clearly evident that pteroylglutamic acid is elaborated as a product of an enzyme system or systems for which *p*-aminobenzoic acid is a substrate. Sulfadiazine competes with *p*-aminobenzoic acid in this system, and it, therefore, must owe much of its effect on growth to the inhibition of synthesis of pteroylglutamic acid. If this product of the inhibited reaction is added to a culture of bacteria which is being retarded by sulfadiazine, then the action of the analog should be overcome and the same amount of pteroylglutamic acid should suffice for one inhibitory dose of sulfadiazine as for a hundred. This is precisely the situation which is found with some strains of bacteria.³ We see, therefore, that, if the structurally dissimilar metabolite is a product of the reaction system which is inhibited by the antimetabolite, antagonism of the action of that antimetabolite may result, even though no direct structural analogy exists between the two substances.

This same situation may be seen with certain other dissimilar antagonists where a like metabolic relationship is clearly evident. As an example, consider the case of cysteic acid. This structural analog of

³ The antagonism of pteroylglutamic acid for sulfanilamide can be demonstrated only with selected strains of microorganisms. Why it does not occur with all species is unknown.

aspartic acid inhibits the growth of *Escherichia coli*, and this action is antagonized competitively by aspartic acid. It is also antagonized, and non-competitively, by pantothenic acid, so that when this vitamin is present no amount of cysteic acid is toxic (24, 253). At first glance, pantothenic acid bears little structural resemblance to cysteic acid, but it is fairly well established that pantothenic acid is synthesized *in vivo* by uniting β -alanine with pantoic acid. β -Alanine is known to arise in bacterial metabolism by the decarboxylation of aspartic acid. If this decarboxylation is being inhibited by the antimetabolite cysteic acid, then no β -alanine for the synthesis of pantothenic acid will be available, and the organism will be rendered deficient in this essential growth factor. However, if pantothenic acid is now added to the culture, the deficiency is relieved, and growth may resume in the presence of otherwise inhibitory concentrations of the analog. Of course, if β -alanine is needed for other essential reactions, the addition of pantothenic acid alone would not be expected to allow growth to begin in the presence of cysteic acid. Fortunately, however, for the demonstration, extra needs for β -alanine did not seem to exist.

Many other instances of non-competitive antagonism between a product of a reaction system and an analog of the substrate for this system are known. For example, glutamine will antagonize non-competitively the effects of methionine sulfoxide, whereas glutamic acid does so competitively (63, 64, 65, 254). Glutamine is the product of the reaction which is inhibited by the analog of glutamic acid.

In all such examples which are worthy of our serious consideration, the product of the enzyme system is clearly the product of the inhibited reaction, and this fact has been demonstrated by actual experiments to show substrate-product interdependence. We come now to consider a second kind of situation in which the structurally dissimilar antagonist is said to be the product of an inhibited enzyme system merely because it does show the antagonism.

Inhibition analysis

This term has been applied to these attempts to postulate metabolic interrelationships by study of compounds of natural occurrence which will antagonize non-competitively the harmful effects of a structural analog of one given metabolite. The cysteic acid example of the preceding section might be taken as the ultimate simplification of this hypothesis, because clearly β -alanine and pantothenic acid arise from aspartic acid. The more complex situations to which inhibition analysis has been applied principally may be illustrated best by consideration of the example from which it arose (234, 235, 253).

The antibacterial action of sulfanilamide may be overcome not only by *p*-aminobenzoic acid but also by purines, or by methionine, or sometimes by pteroylglutamic acid. The only one of these naturally occurring substances which will antagonize the effects of sulfanilamide competitively is *p*-aminobenzoic acid, and this is the only one which is clearly a structural analog of the harmful agent. The other antagonists do not act in a strictly competitive fashion so that, if large doses of sulfanilamide are used, methionine or xanthine will not prevent the toxic effects. If no antagonist is present in the medium, only a small amount of sulfanilamide is needed to prevent growth. If methionine is the only antagonist present, then considerably more sulfanilamide must be added before retardation of growth results, but, once this level of the antimetabolite is reached, more methionine will not antagonize it. However, if xanthine (or sometimes a related purine) is present in addition to methionine, then a second increase in the amount of antimetabolite is required in order to inhibit growth. However, even with optimal methionine plus xanthine, sulfanilamide is still toxic, although much more is required than in the absence of these metabolites. If now, several other amino acids and pteroylglutamic acid are present in the basal medium, the toxicity of sulfanilamide is once again reduced. If, therefore, the minimal effective dose of sulfanilamide is plotted against the composition of the medium, a series of inflections in the curve will be found, each one corresponding to the introduction of methionine, purines, etc. The order of addition of these antagonists is important because in the absence of methionine the effect of the purines is reduced or abolished. Likewise, in *Escherichia coli*, methionine, xanthine, and serine must all be present before the effect of pteroylglutamic acid can be shown (438).

According to the hypothesis which Shive and his collaborators (235) have termed inhibition analysis, these facts are to be viewed as follows. The metabolite *p*-aminobenzoic acid is a substrate for several enzymes and not for just one. Of this family of enzymes the one which is most susceptible to combination with the antimetabolite sulfanilamide is one for which the immediate or remote product is methionine. Therefore, when this product is supplied the inhibition of the enzyme which sulfanilamide affects is no longer of any consequence. The next enzyme which the antimetabolite inhibits is the one for which the product is xanthine or a related purine. If xanthine is present in the medium, then the retardation of this enzyme is of no consequence to growth and therefore enough sulfanilamide must now be added to affect a third enzyme, less susceptible than the previous two, if its toxicity is to appear. A calculation of the inhibition index between the *structurally*

similar metabolite and its toxic analog has been used to detect these postulated products of the reactions. If the index is greater when it is determined in the presence of the structurally dissimilar antagonist, that antagonist is concluded to be such a product. In the case of *p*-aminobenzoic acid and sulfanilamide, the inhibition index is greater in the presence of methionine, and again still greater when purines are in the test system, and so methionine and the purines are deduced to be products of reactions involving *p*-aminobenzoic acid. Mathematical equations expressing this way of stating the idea have been developed (235, 253). The non-competitive antagonists of the antimetabolite are therefore viewed as products of enzyme systems for which *p*-aminobenzoic acid is a substrate, and the order of effectiveness of these antagonists is postulated to indicate which enzymes are more sensitive. In order to fit this hypothesis to the experimental findings, it became necessary to regard some of the non-competitive and structurally dissimilar antagonists as precursors of the substrate rather than as products. It was also occasionally necessary to postulate other rules for some of these substances, because quite obviously all the compounds which might affect the activity of a given antimetabolite were not products or even precursors intimately related to the analogous metabolite. Nevertheless, the results of inhibition analysis have been used by some investigators to postulate details about the route of formation of amino acids, nucleic acid derivatives, and other metabolites.

The general idea of inhibition analysis has been useful and productive in the understanding, or at least the rationalization, of complex relationships, such as that involving sulfanilamide and methionine and the purines. Since it was first put forward by Harris and Kohn (234) for this particular case, and elaborated and extended by Shive and collaborators (235, 253), it has been directly responsible for stimulation of investigations into biosynthetic mechanisms. This aspect of antimetabolites will be discussed in Chapter 10. However, an interesting example of how it may help in the understanding of complex biological situations is to be found in the following. The *p*-nitrosulfanilide corresponding to pantothenic acid, namely, pantoyltaurine-*p*-nitroanilide, is inhibitory to the growth of certain bacteria, and this effect may be reversed by the vitamin in some species. The same substance also has an action on isolated strips of rabbit intestine (437). When such strips are bathed with choline and adrenaline, they contract, because acetylcholine is formed from the choline in the system. Exogenous acetylcholine will elicit a similar response. If the pantothenic acid analog is present, the contraction is inhibited very considerably. This is readily understood, because the synthesis of acetylcholine is brought

about through the intervention of coenzyme A, which is a conjugate of pantothenic acid. Pantothenic acid and its analog probably compete with each other in an enzymic system of which the final product is coenzyme A, and the analog, if present in sufficient amount, is able to prevent the reaction. The pharmacological effect in the intestinal strips may then be pictured as arising from an inhibition of coenzyme A synthesis, which, in turn, retards the formation of acetylcholine from the choline administered. If this explanation is correct, then addition of coenzyme A should nullify the effects of the analog and allow the usual contraction of the muscle. This actually has been found to occur (439). The real complexity arises because pantothenic acid, which should also show this antagonism, does not. Because it was the coenzyme and not the structurally analogous pantothenic acid which exhibited the antagonism to the drug, the conclusion was reached that the latter was acting as an antimetabolite of coenzyme A, and not of pantothenic acid. Application of the ideas of inhibition analysis might well clarify the situation and show that the analog actually was preventing the formation of coenzyme A from the vitamin to which it is structurally related, rather than antagonizing directly the action of the coenzyme which it more distantly resembles in constitution. The failure of pantothenic acid to overcome the effects of the drug in the intestinal strips would thus be another example of the situation described in Chapter 2 in the section on non-competitive antagonism. The metabolite cannot antagonize the antimetabolite, even though a dissimilar metabolite (coenzyme A) can. If the view of the mechanism just proposed is correct, then coenzyme A should be a non-competitive antagonist of the *p*-nitrosulfanilide, whereas, if the postulate is right that it competes with the coenzyme rather than with the vitamin, the antagonism should be competitive. A simple experiment could thus clarify the underlying mechanism. The criterion of competitive versus non-competitive type of antagonism may thus throw considerable light on these complex situations even though it is not the sole criterion in such a study (see Chapter 2).

Although the basic ideas of inhibition analysis have been helpful in understanding experimental observations, the method has some decided limitations. For example, considerable question has arisen about the validity of deductions concerning sequences of reactions arrived at in the manner outlined for the *p*-aminobenzoic acid case. Although inhibition analysis would indicate that the formation of methionine is the first reaction, or set of reactions, to be affected by sulfanilamide, and that synthesis of purines, of other amino acids, and of folic acid are influenced in that order, accumulating evidence (not yet conclusive,

however) points to the formation of folic acid as a primary step, and to involvement with purine synthesis as a secondary effect arising from lack of this metabolite. Another limitation of inhibition analysis is the clear demonstration that a substance which antagonizes the action of a toxic agent is not necessarily a product of a metabolic reaction in which the agent acts as an antimetabolite. Thus, as will be discussed in more detail in Chapter 5, Zn^{++} is a structurally similar, competitive antagonist of the metabolically essential Mn^{++} (256). Magnesium ions, however, will antagonize the action of zinc ions. It is relatively certain that magnesium ions do not arise as a product of a metabolic reaction of manganese ions, as rigorous application of inhibition analysis might indicate. Similarly, untenable deductions which might result from direct application of inhibition analysis to results with toxic synthetic thymine analogs have been noted (255). In its present form, the idea would seem to be a useful guide, but not a sure indication.

The importance of regarding the metabolite as a substrate in an understanding of the action of antimetabolites

In the favored hypothesis, the metabolite was regarded as a substrate with which a specific protein (or other large molecule) reacts. A complex is thus formed which may either be relatively stable, as in the case of oxyhemoglobin or of the biotin-avidin compound, or which may undergo a reaction to yield a new product and the original specific protein, as in the enzymic transformations. This aspect of the mechanism is important. The antimetabolite brings about its effect by competing with the metabolite for the formation of this complex.

Another, and apparently erroneous, idea has sometimes been held and has actuated several investigations. Because many of the essential metabolites bring about their actions by conversion to coenzymes which then form part of an enzyme system, the antimetabolites have been considered to compete with this coenzyme in the enzyme system, and not to be directly concerned with the substrate. Thus, because nicotinamide is built into cozymase (i.e., diphosphopyridine nucleotide), and because this coenzyme then functions in the dehydrogenase system, antagonistic analogs of nicotinamide have been expected to inhibit the dehydrogenase system. Similarly, because pantothenic acid is built into coenzyme A, and because this coenzyme functions in the system which acetylates choline, antimetabolites of pantothenic acid have been expected to inhibit the enzymic synthesis of acetylcholine.⁴ Actually,

⁴ In unfractionated test systems, such as rabbit intestines, some analogs of pantothenic acid do inhibit the synthesis of acetylcholine. This matter was discussed in

however, no specific (i.e., competitively reversible) inhibition is produced in either of these cases. The nicotinic acid analogs probably interfere with the synthesis of cozymase from nicotinamide, and the pantothenic analogs with the synthesis of coenzyme A. Indeed, this latter point has been clearly demonstrated (257). On the other hand, one should look for an analog of "active" acetate or of choline if one wishes to inhibit the synthesis of acetylcholine in isolated systems by means arising from the phenomenon of antimetabolites. A suitable analog of coenzyme A might be expected to displace it from the holoenzyme and thus indirectly to inhibit the synthesis by disorganizing the enzyme. The distinction between substrate and coenzyme in a series of enzymic processes may frequently be clear only if detailed knowledge is available. Certainly, when cozymase unites with the apodehydrogenase we have a specific protein forming a complex with a specific substrate. The complex so formed is the product which happens to be an enzyme which now reacts with the new substrate which is to be dehydrogenated. It is perfectly conceivable that a suitable structural analog of cozymase might compete in the formation of the complex between coenzyme and apoenzyme, and thus might inhibit the enzyme system. Analogs of nicotinic acid thus far examined do not seem to be closely enough akin to cozymase to cause this competition.

We may well ask whether the insistence on these rather subtle distinctions is not just splitting hairs. However, the practical result of holding to this view of mechanism is occasionally instructive. One example is that encountered in attempts to discover inhibitors of pathogenic viruses. For a time, antagonistic analogs of the vitamins and some of the purines were tested for ability to inhibit the growth of virus diseases in animal hosts, in the belief that some coenzyme function of derivatives of these metabolites might thus be blocked with subsequent preferential harm to the pathogenic agent. No success attended these efforts. However, with the recognition that the substrate was the proper point to consider in thinking about such inhibitions, and with the discovery of specific substrates for some viruses (e.g., the influenza viruses), only a short time was needed to find an active and selective inhibitor of the growth of these pathogens (258). By use of compounds believed to be analogous in structure to this

the immediately preceding section. However, the intestines have been shown to synthesize coenzyme A as well as acetylcholine, and these two reactions together carry pantothenic acid to the coenzyme stage and the coenzyme then by a new series of transformations brings about the formation of the hormone. The pantothenic acid analog was pictured as affecting the first in this chain of reactions, and so only indirectly influenced the elaboration of acetylcholine.

substrate of the virus, the activity of the pathogen could be restricted. It seems probable that other such tangible results may be found by other applications of the basic principles.

In the discussion of mechanism, the idea has been implicit that the antimetabolite exerts its effect by inhibiting the *utilization* of the structurally similar metabolite. Most of the existing information is compatible with this view. However, the statement is occasionally made that the analog inhibits the *synthesis* of the related metabolite. No direct evidence in support of this view has been presented. Until such evidence is found, it would seem wiser to look for the explanation in terms of utilization rather than of synthesis of the metabolite.

Facts not readily explainable by the favored hypothesis

All the existing data about the action of antimetabolites on living cells cannot be explained beyond equivocation by what has been called here the favored hypothesis. The discrepancies can be fitted to the hypothesis easily in several instances but with less satisfaction in others. The complexity of living organisms frequently leaves much room for disagreement about interpretation. The following example will illustrate one of the difficulties.

Both pyriethamine and oxythiamine antagonize the action of thiamine in mice, and their toxic effects can be overcome with the vitamin. Both have been shown to inhibit the synthesis of cocarboxylase from thiamine in isolated enzyme systems. If this enzymic retardation is the mechanism of action of both analogs, then their biological effects should be qualitatively identical. They are not. Oxythiamine does not elicit the train of pharmacological signs which is seen with pyriethamine, but rather causes only retardation of growth rate, inanition, and death (431). An obvious explanation of this difference is that other reactions in addition to the formation of cocarboxylase are affected by the one analog, but not by the other. Alternately, the one may be fixed or anchored at the site of action, while the other is not. In fact, with pyriethamine direct evidence exists for its accumulation in animals, and this may account for its different behavior. However, these are plausible postulates which have not been proved beyond equivocation. Some other situations are also difficult to understand. Let us take note of some of them.

Stimulation of living organisms by antimetabolites. The stimulation of living organisms by subinhibitory concentrations of several antimetabolites is not readily explainable by the favored hypothesis. Some more or less plausible reason for this stimulation can be offered

which may satisfy the more liberal or imaginative investigators. The more conservative ones, however, may find it difficult to follow, and the future may show that it has little resemblance to fact.

Two related tentative explanations may be given. According to the first, the orderly growth and development of an organism is controlled by a multiplicity of metabolic reactions. Some of these feed raw materials into the system at the proper time and in approximately the proper amount. Others are concerned with the removal of waste materials, many of which may be rather injurious to the living cell. Still others are concerned with the transfer of energy or with the differentiation of morphological structures. These are all so interwoven that one single process may have several of these functions as different aspects of its activity. If now one of these interdependent reactions is partially inhibited by the admission of small amounts of an antimetabolite, other systems may respond by altering their rate of performance. If all but one reaction system of an organism were thus stimulated, and if the inhibited reaction were still functioning sufficiently well so that an absolute deficiency of its products did not result, it is possible to conceive of a transitory general stimulation of growth or development.

According to the second explanation, the products of one set of reactions frequently are found to inhibit other essential processes, and indeed this antagonism between pairs of metabolites will be discussed at length in Chapter 5. The partial deficit of one essential metabolite, such as might be occasioned by small amounts of an antimetabolite, could result in the relieving of such a natural inhibition. This might occur while enough of the metabolite related to the inhibiting agent was still functioning to allow its more direct reactions to be realized. This release of the natural inhibition could result in stimulation of growth.

The actual proof of the validity of such explanations would be rather difficult with techniques now available. Although the postulates have some merit, the existence of the stimulation raises a serious question as to the adequacy of the favored hypothesis about mechanism of action of the antimetabolites.

Relationship of nutritional requirement of an organism to the activity of an antimetabolite. This correlation as it was set forth in Chapter 2, is difficult to explain with the hypothesis proposed in this chapter. Obviously, one can assume a lack of absorption of the antimetabolite by those cells which do not have a need to take up the re-

lated metabolite (i.e., those which do not require the metabolite). Such postulates are not possible to verify experimentally. Perhaps an extension of the hypothesis about mechanism of action of the anti-metabolites will be required to clarify this aspect of the problem. The explanation may, on the other hand, be found in other aspects of biochemistry such as the metabolism of the vitamins and may then fit nicely into the present hypothesis.

Antagonism between pairs of structurally similar drugs. In the chapter dealing with applications to pharmacology it will be shown that two structurally similar compounds may act antagonistically in an organism even though neither of them is found in the organism naturally. The two structural analogs, neither of which is a metabolite, thus exert an antagonistic action to each other in a foreign metabolic field. Morphine and allyl normorphine constitute such a pair. Neither of these substances occurs in a dog, and yet the toxic manifestations of morphine may be overcome completely by giving allyl normorphine to dogs narcotized with the former drug (259). Likewise, salicylic acid and *p*-aminosalicylic acid, neither of which has been found in acid-fast bacteria, are mutually antagonistic when both are present in the medium in which such organisms grow (260).

It is difficult to explain why this should be so by use of the favored hypothesis. Presumably, if a metabolite were being displaced from its normal pathway in the dog by the presence of morphine, then another structural relative, namely, allyl normorphine, might displace the morphine but still leave the normal metabolite excluded from its usual reaction by the presence of this new competitor.⁵ It may be that we do not sufficiently understand a three-cornered competition and thus have no clear picture of this situation. More study of the effects of two inhibitors, acting simultaneously with a metabolite on a single protein *in vitro*, might make understandable this competition of structurally similar drugs, but such studies have not yet been made. On the other hand, the two antagonistic drugs may participate in more than one reaction, and to differing degrees. If this were so, a logical

⁵ In order to clarify the argument, the assumption has been made that morphine acts on animals by antagonizing the effects of a structurally related metabolite. There is no factual basis for this particular assumption. If it is true, then the argument is as outlined in the text. If the assumption is not true then the whole situation is removed from the field of antimetabolites. This point will be discussed at greater length in Chapter 8. However, in the second example cited in the above, namely the antagonism of salicylic acid and *p*-aminosalicylic acid, the metabolite, namely *p*-aminobenzoic acid, is known to exist.

explanation of their behavior might be constructed. At present, however, the situation presents a challenge to the favored hypothesis.

The desirability of using isolated enzyme systems in attempts to resolve such difficulties

Whether or not the discordant findings mentioned in the preceding section constitute a serious threat to the acceptance of the favored hypothesis cannot be determined readily by studies on intact living organisms. The questions of permeability of the cell membrane and of concomitant destruction of the administered antimetabolite arise constantly to becloud the issue, as we have already seen. Therefore, the only direct means of testing the validity of the postulates about fundamental mechanisms would seem to lie in the study of isolated enzyme systems in which the possibility of these extraneous reactions is minimized. If subinhibitory concentrations of an antimetabolite were found to stimulate such an isolated enzyme system with which the related metabolite acted as substrate, then the favored hypothesis would be open to serious question. So long as the stimulation is observed as an effect on an intact organism, it can be partially explained, possibly along the lines already outlined above.

Another advantage to be gained by the use of isolated systems is that the conversion of one metabolite into another is largely eliminated. In living cells, the concentration of substances such as amino acids is subject to uncontrollable variation, due to the ability of the living tissue to synthesize some of these metabolites or to convert them to other materials. In an isolated enzyme system this uncertainty about amounts actually present is usually absent and therefore features of the underlying mechanism of action of an antimetabolite are easier to distinguish.

Other hypotheses about the mechanism of action of antimetabolites, and their possible relationship to the favored hypothesis

Several postulates have arisen at various times to explain the mode of action of antimetabolites. Most of these have dealt only with sulfanilamide and its derivatives because of the practical usefulness of these drugs in therapy. Several of these do not now enjoy widespread acceptance because they are at variance with some outstanding experimental observation or at least have not been reconciled to all the facts to the satisfaction of many investigators. The hypothesis outlined earlier in this chapter seems to account for the observations better than others which have been advanced and has, therefore, been called the favored hypothesis. Some of the different postulates sometimes

illuminate this one rather than controvert it. One of these is of particular interest.

Sulfanilamide or sulfathiazole react in a test tube with an aqueous solution of reductone to give a compound in which the amino group of the drug has coupled with the carbonyl of this hydroxyaldehyde. *p*-Aminobenzoic acid likewise will react with this aldehyde to form a similar compound. When *p*-aminobenzoic acid is added to a solution of the sulfathiazole-reductone compound, the metabolite displaces the drug, and the *p*-aminobenzoic-reductone compound is found (440). Reductone was chosen for these *in vitro* experiments because colorimetric tests had indicated that it or a similar carbonyl compound was formed by bacteria in the early phase of their growth. Because sulfonamide drugs do not usually kill bacteria, but rather inhibit their multiplication after a few cell divisions have occurred, the reaction of the drugs with a product such as was indicated to appear in the early stages of growth was considered to be significant. It was suggested (440) that *p*-aminobenzoic acid, which unquestionably exists in bacterial cultures, formed a complex with reductone (or whatever the carbonyl compound was) and conserved it from destructive processes so that it was available for continued cell multiplication. In addition, this complex was believed to facilitate essential reactions in the cell. Folic acid, for example, might possibly be formed from it. Since *p*-aminobenzoic acid could, *in vitro*, displace the drugs, and thus bind the reductone, their mode of action in retarding bacterial growth was pictured as a trapping of reductone with consequent deleterious effects.

Sufficient information is lacking from which to decide whether the need for reductone is why bacteria stop multiplying in the presence of sulfanilamide. Considerable elaboration of the hypothesis may be necessary before it can account for all facts, but conceivably this could be done. The *in vitro* demonstrations of displacement by *p*-aminobenzoic acid of a sulfonamide drug from its combination with reductone do, however, fit well with the favored hypothesis. They constitute a laboratory model in which a simple aldehyde can be viewed as taking the place of a specific protein. It shows again that such displacements can occur under physiological conditions. The molecule with which the combination takes place may be an enzyme or other specific protein, and it is even possible that the union is through a carbonyl group. In certain cases, the specific reactant may be a simple molecule such as reductone, rather than a protein. As Bell et al. (440) suggest, this complex may be just what is required for the next step in a biosynthetic process.

Hypotheses about mechanism arising from the alteration of growth of crystals by isomorphic substances

As pointed out in the preceding section, the necessity of participation of a specific protein in explanations of the action of antimetabolites has not received universal support among biochemists. Particularly among European investigators this has seemed an unessential complication, and attempts have been made to account for all the experimental facts on a simpler basis. Some of the most impressive evidence for such ideas of mechanism rests on phenomena of crystal growth. The relationship of this evidence to the mechanism of action of the antimetabolites has been urged first by Erlenmeyer et al. (10).

If a simple inorganic substance be crystallized from a solution which also contains a compound which crystallizes in a form isomorphic in two dimensions with the first substance, then the growth and shape of the crystals which are formed may be altered considerably. For example, sodium chloride ordinarily crystallizes in cubes. However, if urea is added to the solution, the sodium chloride is then obtained as octahedra instead of as cubes. Two-dimensional isomorphism in the crystals of the two "competing" substances seems to be a requisite. In many of these cases one may say that the growth of the crystals of the one compound has been inhibited or altered by the presence of the second of similar physical structure. This might seem to be the ultimate simplification of the superficially similar phenomenon of the antimetabolites, in which living organisms, and even their characteristic protein constituents, have been eliminated from consideration.

Other interesting situations in crystallography have been held up as models for the understanding of the mode of action of the antimetabolites (261). Thus, when crystals of sulfanilamide or of *p*-aminosalicylic acid are sublimed to a freshly cut (100) face of sodium chloride, the crystals of the sublimate orient themselves in definite order with respect to the receiving surface, and this order is similar for these two different analogs of *p*-aminobenzoic acid. When this metabolite is similarly sublimed, the orientation of its crystals is in a different plane from those of its antimetabolites. When the receiving surface is not the (100) face of sodium chloride, but is merely glass, no orientation of the crystals of the sublimate is observed. These facts may be seen from the photographs of Figure 5 which have been taken from the work of Erlenmeyer and Müller (261).

To the author of this monograph this crystallographic information would seem to supplement rather than to exclude the favored hypothesis. The information gained by studying crystals may well serve

to give a clearer insight into the mode of combination of metabolite and its specific reactant, which is usually a protein. It may also help

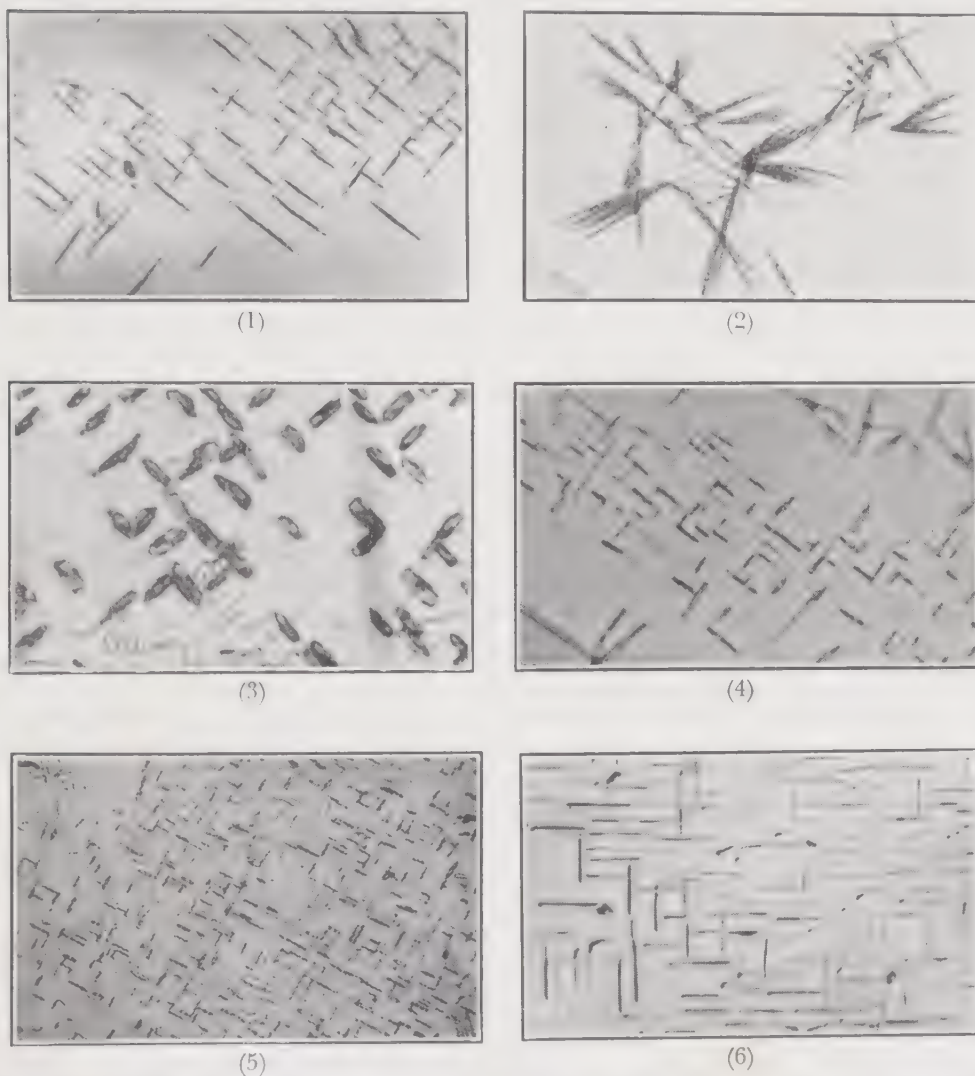


Figure 5. Orientation on a crystal surface of sublimates of *p*-aminobenzoic acid and of antimetabolites related to it. 1. *p*-Aminobenzoic acid sublimed to a fresh (100) surface of NaCl. 2. *p*-Aminobenzoic acid sublimed to a glass surface. 3. *p*-Aminosalicylic acid sublimed to a fresh (100) face of NaCl. 4. Salicylic acid sublimed to a fresh (100) face of NaCl. 5. Benzoic acid sublimed to a fresh (100) face of NaCl. 6. Sulfanilamide crystallized on a fresh (100) face of NaCl. (Reproduced with the kind permission of Professor Erlenmeyer and *Helv. Chim. Acta.*)

to understand why the analog of the metabolite is able to compete for this same site of combination on the protein. The specificity of the action of a great number of antimetabolites, however, would make it

seem difficult to explain all the observed facts on the basis of a simple crystallographic model without the participation of a characteristic protein in the organism. Certainly, the competition of metabolite and antimetabolite for a place on a specific protein has been demonstrated directly with oxygen, carbon monoxide, and hemoglobin, and with biotin, biotin sulfone, and avidin. These findings may, however, be couched in the terms of the crystallographic view. The future elaboration of the "more direct" hypothesis may make it more acceptable than the favored one. At the present, however, the favored hypothesis seems to account for existing data better than any other postulate. It may even prove to be the true explanation.

Summary

Antimetabolites are pictured as acting by excluding a structurally related metabolite from combination with its normal specific reactant. This latter is usually an enzyme so that the antimetabolite, by preferentially attaching itself to it, excludes the metabolite and thus prevents the formation of an enzyme-substrate complex. The specific reactant is not necessarily an enzyme and may be some other large molecule such as hemoglobin, apocarboxylase, or an antibody. It may even be a small molecule with which the metabolite normally combines. The antimetabolite unites with this specific molecule because it possesses most of the features which allow the metabolite so to react. The result of this exclusion is manifested in living organisms or in parts of them as the signs of deficiency of the metabolite. Since living creatures are complex and the reactions which they conduct are at best poorly understood in detail, all aspects of the action of a given antimetabolite may not be clarified by this basic postulate. Examples of this have been discussed. Some alternative hypotheses have likewise been indicated. The displacement idea has been favored because it seems to explain adequately more of the existing data than do others, but its final validity is not yet fully established in all cases.

The spectrum of activity of antimetabolites

Inspection of existing data shows clearly that there is a spectrum of activity of the antimetabolites, ranging all the way from ability to act as the metabolites do down to possession of strong antagonistic potency. Let us examine several aspects of this situation so that we may better understand the basic phenomenon.

Metabolite and antimetabolite activity of the same compound in the same species

Frequently one finds that a single antimetabolite may show strong antagonistic effects when it is tested at one range of concentration but may then act in place of the metabolite at higher ranges of concentration. This situation prevails even though the testing is done with the same organism, and hence it is not to be explained as a variation among species. For example, *p*-aminobenzamide in rather small amounts inhibits the growth of several kinds of bacteria, and this effect may be overcome by increasing the concentration of its related metabolite, *p*-aminobenzoic acid, in the culture. However, when larger quantities of the aminobenzamide are tested for ability to replace *p*-aminobenzoic acid as a growth factor for bacteria, the compound is found to have such activity (37). At first glance one may attempt to explain this type of behavior by assuming that small amounts of *p*-aminobenzoic acid are formed by hydrolysis of the *p*-aminobenzamide. Although this explanation may be adequate for the example just described, it is not sufficient for many others. A somewhat clearer example is that of α -methylpantothenic acid. When this substance is tested with *Lactobacillus casei* it is found to inhibit growth in competition with panto-

thenic acid. However, when the analog is assayed for pantothenic acid potency as a growth factor for this same bacterium, it is found to possess such activity at high concentrations. The amounts of the analog needed to show pantothenic acid potency are greater than those which antagonize the growth-promoting effect of small concentrations of the vitamin. As a result, if increasing quantities of the analog are tested in the presence of a small (and suboptimal) amount of pantothenic acid, one sees first inhibition of growth, followed by stimulation. These facts are illustrated by the data in Table 1 which have been

Table 1

Growth-inhibitory and growth-stimulatory properties for *Lactobacillus casei* of α -methyl pantothenic acid

Pantothenic Acid, gammas	Analog, gammas	Growth, milligrams
0.05	0	25.8
0.05	1	24.8
0.05	5	19.3
0.05	10	17.7
0.05	50	11.2
0.05	100	8.2
0.05	200	10.6
0.05	500	11.3
0.05	1,000	11.8
0.20	0	75.2
0.20	100	32.0
0	0	3.0
0	100	7.2
0	500	8.7

taken from the work of Pollack (190). The range for antimetabolite activity overlaps slightly that for metabolite effect, so that complete suppression of growth is not achieved. In this instance there is no possibility of pantothenic acid itself arising from or contaminating the analog. Similar vitamin activity has been found for large amounts of other analogs of pantothenic acid (193). In smaller concentrations these same substances are antagonistic to the vitamin.

Behavior of this sort has been found with animals as well as with bacteria. Thus, the *p*-nitrobenzyl ether of N-acetyl-diiodotyrosine, a relative of thyroxine, antagonizes the action of this hormone on tadpoles (128). This may be seen from its ability both to protect against lethal doses of the hormone as well as to inhibit the accelerated metamorphosis caused by thyroxine. With minimal lethal doses of the

hormone, the analog is able almost completely to nullify its effects. However, about twice this amount of the analog when tested in the absence of thyroxine, has some hormone action, and this is evidenced by a stimulation of the rate of metamorphosis of the tadpoles. Obviously, the proximity of the ranges of concentration for demonstrating antimetabolite and metabolite properties may cause some masking of the two actions, just as it did with α -methylpantothenic acid.

Cases such as these differ from the situation discussed in Chapter 2 under the heading of stimulation of living organisms by antimetabolites, because in those instances the stimulation was brought about by subinhibitory amounts of the antimetabolite, whereas here the effect is with superinhibitory quantities. It would seem that some antimetabolites have a weak but inherent metabolite potency which is apart from their antagonistic properties. Furthermore, it would seem that the antimetabolite action does not completely interfere with the expression of the prometabolite effect. If it did, the prometabolite effect would not be seen.

The metabolite potency of some antimetabolites when tested in new species

Most antimetabolites have not been found to exhibit the perplexing duality described in the preceding section. However, with several, they may act as an antimetabolite in one species, and as the related metabolite in others. If a sufficient number of kinds of living things are examined, all grades of activity ranging from strong antagonistic or antimetabolite action to weak metabolite potency may be found. For example, *p*-aminosalicylic acid (2-hydroxy-*p*-aminobenzoic acid) is a rather strong inhibitor of the growth of the tubercle bacillus, and in this organism its effects are overcome with small amounts of the metabolite *p*-aminobenzoic acid (260). For many other kinds of bacteria, it is a much weaker injurious agent, but nevertheless it does cause inhibition of growth. However, when it is tested on mutant strains of *Escherichia coli* which require *p*-aminobenzoic acid as a growth factor, the analog is not harmful, but rather has about one-sixth the potency of the metabolite which it effectively replaces (262).

A somewhat related situation has been found with oxybiotin. When the sulfur atom of biotin is replaced by an oxygen, oxybiotin is obtained, and this substance is quite active as a growth factor for microorganisms and higher animals in place of the natural metabolite (263, 264). For some species it is practically equal in potency to biotin. For others, however, it is only about one-tenth as effective, and for still others, it is practically inert (265). Some *Clostridia* have even been re-

ported to be injured by oxybiotin, although this effect has not been shown to be reversible by biotin. Thus in this biotin analog one can see the progression from full ability to replace the vitamin down to complete inability to do so and even to harmful action. An even clearer situation of this sort is found with two other close relatives of biotin, namely homobiotin and norbiotin (408). For some yeasts these substances show biotin activity, whereas for others they act as injurious materials, the effects of which may be counteracted by biotin.

The explanation of such cases may be somewhat as follows. In general, as we have seen in Chapter 3, essential metabolites are very exacting in their structural requirements. Only a single molecular species, and not even closely related compounds, have the ability to act as the metabolite. This specificity is not always so exquisite, even though the smallness of the number of active relatives of the metabolite is striking indeed. Most of the structural relatives interfere with the normal functioning of the metabolite, as we have seen in Chapter 3. They are thus antimetabolites. The ability of species to use a close structural relative as a substitute for the metabolite varies, probably because of differences in the specific proteins with which the metabolite reacts. The variability in combining power for oxygen of the hemoglobins (232, 252) of diverse species helps in the understanding that this may be so. Furthermore, if the metabolite has a number of functions and is thus concerned with several proteins, the structural specificity needed for successful reaction with each may not be the same. With some, oxybiotin may function as well as biotin does, whereas with others oxybiotin may not do at all. If two species differ in that one lacks these latter metabolic reactions of biotin, then the growth-factor activity of desoxybiotin for one and not for the other can be envisioned. One can thus picture how oxybiotin may be acceptable to *Saccharomyces*, and totally objectionable to *Clostridium*. The latter would thus appear to be the more fastidious. Similarly, the tubercle bacillus may be completely unable to use *p*-aminosalicylic acid in place of *p*-aminobenzoic acid, whereas strains of *E. coli* can utilize it with but slight difficulty.

Other examples are known of the deciding role of the species in determining whether metabolite or antimetabolite action of an analog will be manifested. Thus ethylpyridoxine is capable of replacing pyridoxine as a growth factor for tomato roots, but the same compound is antagonistic to this vitamin in the growth of certain fungi (266, 267). Ethylpyridoxine is 2-ethyl-3-hydroxy-4,5-dimethylolpyridine and differs from pyridoxine in having an ethyl rather than a methyl group in position 2.

The metabolite activity of a few antimetabolites is apparently due to conversion of them to the metabolite. Those species which lack this ability may then be susceptible; those which have it may resist with impunity. For example, desthiobiotin is a powerful antagonist to biotin in *Lactobacillus casei* but has biotin activity for *Saccharomyces* (50, 51, 52). The latter organism has been demonstrated to insert a sulfur atom into desthiobiotin and thus to form biotin from it. Similarly, strains of *Endomyces vernalis* have been found which are not only resistant to pyrithiamine, but which can use it in place of thiamine to meet their growth requirements (120). Here it has been shown that the resistant strains possess an enzyme system which cleaves pyrithiamine into its pyrimidine and pyridine halves by rupture at the methylene bridge. The pyrimidine moiety so formed is identical with the one from thiamine, and *Endomyces* can use this in place of the vitamin itself. Although some of the cases may thus be explained on the basis of conversion of the antimetabolite to the metabolite, others seem incapable of elucidation in this fashion because of the nature of the chemical change which would be required. For example, by direct measurement it has been shown that bacteria which can use oxybiotin instead of the naturally occurring vitamin do not convert it into biotin as a preliminary to utilization (268). For this and similar cases other postulates such as the ones in the foregoing paragraph must be explored.

Partial replacement of a metabolite by analogs which by themselves are inert

Analogs of some of the vitamins are known which have neither growth-factor nor growth-inhibitor activity. However, when they are tested in the presence of suboptimal concentrations of the metabolite, they have stimulatory activity. For example, the analog of riboflavin produced by replacement of the ribityl side chain by an arabityl group has no riboflavin activity for *Lactobacillus casei* when tested by itself. However, when assayed in the presence of small amounts of the vitamin, it does show a fair degree of riboflavin activity (269). The same is also true for the analog of pantothenic acid in which the secondary alcoholic group has been exchanged for an amino group (270). In these situations, the logical explanation would seem to be that the metabolite has several functions in the organism. For some of them, only the metabolite itself can suffice, but, for others, a very closely related substance can be made to function. The examples which are known have always involved an analog which is exceedingly closely related in structure to the vitamin. Thus, if suboptimal quantities of

riboflavin are supplied the organism may use these for its most exacting functions, and for the others the arabityl analog may serve almost as well. For this reason the analog has metabolite potency in the presence of the vitamin but not in its absence. If this view of the matter is correct, the possibility of constructing analogs to interfere with one but not all of the functions of a metabolite seems worthy of consideration. Desirable selectivity of action as between organisms or types of tissues of the same organism might thus be achieved. For example, if one type of tissue possessed only the most exacting functions of the metabolite, whereas other parts of the organism used the less exacting ones as well, an analog could be conceived which would specifically harm this type of tissue without destroying completely the whole individual. In any event, this furtive metabolite activity of some structural analogs is one aspect of the subject of this chapter.

Progression from metabolite action to antimetabolite potency with graded change in structure

In an homologous series of compounds, some may show metabolite activity, whereas others may be potent antimetabolites. One of the best examples of this is seen with analogs of thiamine in which the sole structural change has been made in the alkyl side chain at position 2 of the pyrimidine ring. Thiamine itself has a methyl group at this point, and, of course, has full vitamin potency. The corresponding ethyl analog still has vitamin activity amounting to about 10 per cent of that of the natural metabolite. However, the butyl compound is an antimetabolite as shown by its ability to cause the signs of thiamine deficiency in rats (7). This case of the alkyl homologs of thiamine is particularly instructive because in it one can see the submergence of metabolite activity followed by emergence of antagonistic effect as one goes up the homologous series. Usually, as slight alterations of structure are made, one passes from the metabolite to a strong antagonist without the intermediate stop of a slightly active relative.

An even better example of how metabolite activity may fade and be followed by strongly antagonistic effects is found among derivatives of succinic acid. Monodeuterosuccinic acid, which differs from the metabolite only in having one hydrogen atom replaced by an isotopic atom, is attacked by succinic dehydrogenase only 70 per cent as readily as is succinic acid itself. Dideuterosuccinic acid is only 40 per cent as effective as is the normal metabolite (271). If a methyl group be used in place of one α -hydrogen atom, then this analog is a strong inhibitor of the enzyme. The exchange of a methyl group for an hydrogen atom would seem to be a small alteration, and yet it has yielded a potent

antimetabolite. The even smaller change involved in the deuterium analogs has allowed demonstration of the intermediate stage of reduced, but nevertheless prometabolite, activity. These are the findings with the enzyme derived from *Escherichia coli* (275). When a different species (horse) is used the situation is not quite the same. With this variety of succinic dehydrogenase, the α -methylsuccinic acid still has some metabolite potency and the ethyl analog has even less. The higher alkyl derivatives are inhibitors of the enzyme (272). We see here, therefore, another example of how the spectrum of activity is brought out in differing living things.

These four aspects of the spectrum of antimetabolite activity one must recognize. Without them, an array of experimental observations might seem rather bewildering. With an appreciation of these features of the phenomenon of antimetabolites, a better understanding may develop of the closeness of essential metabolites and their antagonistic relatives. The borderline between the two may depend merely on concentration, as in the first aspect discussed, or on the particular species in which the test is carried out, as in the second aspect. It may also be decided by minute alterations in the substance, or group of substances being examined.

CHAPTER 5

The natural occurrence of antimetabolites as participants in physiological processes and as etiological agents in disease

In the preceding chapters, mention was made of the natural occurrence of pairs of structural analogs which behaved antagonistically to each other in properly chosen test systems. One can regard some of these cases as examples of naturally occurring antimetabolites and can thus see how nature long ago has seized upon this phenomenon, possibly as an elegant way of controlling natural processes. Sometimes the net result of the antagonism is the production of a disease. This can occur when one species elaborates a compound which is harmful to a second kind of living thing. Among such substances are the antibiotics which are used by man to control the growth of microorganisms. Some evidence exists to show that a few of these antibiotics probably are naturally occurring antimetabolites. They produce disease in the microorganisms they inhibit. Similarly, analogs of other metabolites have been found in nature and demonstrated to play a role in the etiology of a few diseases of plants and higher animals. In addition to these antagonisms between different species we may discern a related situation at work in a single individual. Here two similar metabolites compete with each other. When this occurs, the conclusion seems justified that the antagonism is of importance in normal physiological processes. Let us therefore examine the evidence for this point of view in order to see its merits and shortcomings.

Competition between structurally similar ions

Physiologists have known for many years that antagonisms exist between simple metallic ions such as those of sodium and potassium, and of calcium and magnesium. The antimetabolite view of these antago-

nisms is the first logical explanation of them. Among enzymologists the explanation was beginning to clarify because potassium ions had been recognized as necessary parts of enzyme systems which transferred phosphate groups to glucose (489). Sodium ions had been found antagonistic to potassium in this process and a competition based on structural resemblance had been pictured. About ten years later, 1948, MacLeod and Snell applied the antimetabolite explanation to the understanding of antagonisms between these ions in bacterial growth (291, 292). They recognized the close similarity of some of them to the competition between the essential metabolites and their structural analogs.

Let us take the case of the monovalent metallic ions. Potassium has been recognized for many years as essential to the growth and well-being of many kinds of living things. Bacteria as well as higher plants and animals cannot grow without it. The multiplication of these microorganisms is so dependent on potassium ions that quantitative analytical procedures have been developed for estimation of small quantities of potassium microbiologically. Over a defined range of concentration, growth is directly proportional to the amount of potassium in the medium. Even for isolated enzyme systems potassium ions may be specifically required for activity; e.g., in the phosphorylation of glucose by phosphopyruvate, small amounts of potassium ions are required (489). Little doubt can exist that these ions are essential metabolites just as thiamine and *p*-aminobenzoic acid are.

If *Lactobacillus casei* is grown in a basal medium relatively free of sodium and ammonium ions and supplied with a minimal growth-stimulating amount of potassium ions, then growth can be inhibited by addition of sodium ions, and this effect can be overcome by increasing the concentration of potassium ions. Data to illustrate this point have been taken from MacLeod and Snell (292) and reproduced in Table 1.

Table 1

Molar ratios of Na^+ to K^+ permitting half maximal growth at various levels of added Na^+

Organism	Mg. Na^+ per 10 cc. medium			
	25	50	75	100
	$(\text{Na}^+)/(\text{K}^+)$ for half maximal growth			
<i>L. arabinosus</i>	472	620	725	765
<i>L. casei</i>	530	640	877	895
<i>S. fecalis</i>	340	530	688	805
<i>L. mesenteroides</i> 9135	1,890	2,120	1,990

The antagonism is of the competitive type or nearly so for the four species tested. The inhibition index varied from about 500 with *S. fecalis* to about 2,000 with *L. mesenteroides*. When the atomic structures of sodium and of potassium are compared there can be little doubt but that they are related. Indeed, their position in the same group of the periodic table signifies this. As would be anticipated if the view of sodium ions as antimetabolites to potassium ions is correct, cations of other groups in the periodic table did not antagonize the growth-promoting action of potassium.

When other monovalent alkali ions were examined, some further interesting facts were uncovered. Thus, rubidium, the next element to potassium in group 1 of the periodic table, was able to replace the potassium ion for the growth of *L. arabinosus* and of *S. fecalis*. It also was able to antagonize the effects of sodium ions. For *L. mesenteroides*, however, rubidium could not replace potassium as a growth factor, and, indeed, acted as an antagonist to it. The similarity of this situation to that described in Chapter 4 for the recognized antimetabolites is striking.

If the favored hypothesis outlined in Chapter 3 is correct, these findings with bacteria can be understood in the following manner. Potassium ions are essential metabolites which combine with several specific proteins to yield complexes of the type described as semi-enzymes. These then play an essential role in metabolic reactions necessary for growth. Because of structural resemblance, sodium ions, if present in high enough concentration, can displace potassium. The complex which thus results is unable to function in place of the potassium complex. Increasing the concentration of potassium ions allows the ousting of the combined sodium and thus permits normal metabolism. With rubidium ions, the complex formed with the various potassium-requiring proteins can occur, but the resulting complexes can still serve metabolically in place of the normal products. This is why rubidium can replace potassium in the growth of *L. casei* and *S. fecalis*. However, with *L. mesenteroides*, which cannot grow with Rb^+ instead of K^+ , one would need to assume that a further potassium-requiring protein was essential to growth, and that this one could not use rubidium ions. If this assumption is correct, then Rb^+ should reduce the amount of K^+ required for growth. Experimental trial of this point showed that this was indeed true with low concentrations of Rb^+ . With high concentrations, the inhibition of growth which resulted was probably due to the exclusion of K^+ from this new and more exacting essential protein.

When divalent cations were investigated, manganese seemed to be the one which acted as the essential metabolite for these bacteria, and zinc ions behaved as the antagonist to it. The relationship may be seen from the data in Table 2 which have been taken from MacLeod and

Table 2

Reversal of Zn^{++} toxicity for *L. arabinosus* by Mn^{++}

Mn ⁺⁺ gammas per 10 cc.	Gammas Zn ⁺⁺ per 10 cc.			
	0		400	
	% of incident light transmitted			
	24 hrs.	45 hrs.	24 hrs.	45 hrs.
0	95	95	100	99
1	76	70
10	37	33
100	20	19	99	99
200	99	22
300	97	19
400	27	18

Snell (292). Just as with potassium and rubidium, calcium or magnesium ions could be shown to reduce the amount of Mn^{++} required for growth, although in the absence of Mn^{++} , Mg^{++} or Ca^{++} were inactive. This was interpreted to mean that Mg^{++} could serve equally well as Mn^{++} in some specific reactions of the latter but not in all. In fact, independent evidence for such a concept is found in enzymology where it has been observed that several enzymes which require manganese ions for activity can use other divalent ions almost as well. Although Mn^{++} antagonized the toxic effects of Zn^{++} , Ca^{++} or Mg^{++} were also able to do it. If the explanation just outlined is the correct one for these ion antagonisms, we must then conclude that the specific reactions of manganese ions with which zinc ions compete are the ones for which calcium or magnesium can also serve. The processes involving Mn^{++} which cannot be carried out by Ca^{++} or Mg^{++} are not the ones with which Zn^{++} interferes.

Similar findings have been made with inorganic anions. For example, the nitrate ion is a requisite for some soil organisms which gain their energy by reduction of it to nitrite. For these organisms, chlorate ion is quite toxic, and to a certain extent, this effect is competitive with nitrate (293). Chlorate, however, is probably not a physiological substance normally present in the environment as is the case with sodium ions. By pursuing this same hypothesis about the nature of ion antagonism, it has been possible to show that the toxic effects of selenate in the growth of yeast may be overcome with sulfate (294). Sulfate is undoubtedly a metabolically essential ion, and its structural resemblance to selenate is clear.

Antagonisms between pairs of ions in the growth of plants have been recognized for a long time and have been much studied. If one views the results in terms of the antimetabolite phenomenon, they acquire an understandableness which has not been possible previously. Thus, in the growth of wheat and other cereals, Rb^+ has been shown to antagonize the stimulatory properties of K^+ (409). The relationship of these two ions was competitive, and although the structural resemblance of them was pointed out, many plant physiologists were reluctant to accept this as the underlying reason for the biological effects (410). Now that more is understood about the antimetabolite phenomenon, the objections which were raised can be easily resolved. Another similar situation in the promotion of growth of soybean seedlings has been studied in which it was found that Mn^{++} and Fe^{++} were competitive antagonists (411). When the concentration of Mn^{++} was raised, the plants exhibited characteristic signs of iron deficiency, whereas when Fe^{++} was increased the manifestations of manganese deficiency appeared even though this ion was present in the nutrient solution. The toxicity of each of these naturally occurring ions could not be represented by an absolute value but rather was dependent on the ratio of concentrations of the two substances. The interpretation of this observation is somewhat beclouded because the participating ions may undergo oxidation to a higher valent state. In the days in which these experiments were performed, it was concluded that manganese ions promoted the oxidation of the iron and this led to the formation of insoluble iron compounds. However, an explanation based on the structural resemblance of the two competitors may prove to be more adequate.

Antagonism between testosterone and estrone

Let us now consider the case of the sex hormones which was mentioned briefly in Chapter I. Little doubt can exist that both testos-

terone and estrone (or its derivatives) are metabolites. Deficiencies of either can be induced by surgical operations to remove the gland which produces them. The antagonism between them has been sensed since their discovery, because the induction of female character in normal males with estrogen and, to a lesser extent, of maleness in females with testosterone are well known. In view of this antagonism, it was difficult to understand why normal males should sometimes excrete so much estrogen, and why androgen was found in females. The very close structural resemblance of testosterone and estrone has been

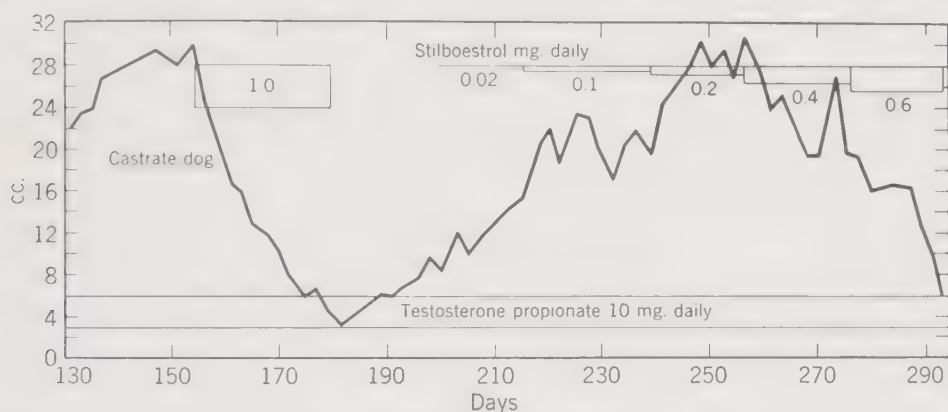


Figure 1. Secretion of prostatic fluid as influenced by androgen and estrogen. (Reproduced through the kind permission of Dr. Huggins and the *J. Exptl. Med.*)

known for almost a generation and can be appreciated from the formulae shown in Figure 16 of Chapter I. What quantitative data can be offered to argue the point of view that they are naturally occurring antimetabolites, one of the other? Most of these data have been assembled from investigations which had little thought of making such a demonstration, but some recent studies have had this possibility much in mind (119).

The measurement of secretion of prostatic fluid in the castrate dog is a good quantitative method for the determination of response to androgen. In such a test system, estrogen suppresses the increased flow of fluid which testosterone would otherwise elicit. This can be seen from Figure 1, which has been reproduced from the work of Huggins and Clark (295). A castrate immature dog had been injected daily with testosterone propionate, 10 milligrams for 160 days, during which time the secretion had risen from 0.1 to 30.2 cubic centimeters. This androgen was continued at the same rate throughout the experiment and in addition stilboestrol injections were begun. Daily amounts of stilboestrol, 1.0 milligram and 0.6 milligram, caused a great decrease of secretion, but dosages of 0.02, 0.1, and 0.2 milligram did not affect the

output. Stilbestrol 0.4 milligram caused a leveling of the rate of secretion. Similarly, injection of estrogen into normal dogs markedly suppressed the usual secretion.

Another test system which has been used is that involving acid-phosphatase levels in the blood. The amount of this enzyme is controlled by testosterone as one can see from a study with castrated males. Estrogen will depress the level of acid phosphatase in the blood, and testosterone will increase it (296, 297). Other measurable biological responses to these hormones have been used to gain more information about the antagonism. For example, testosterone will cause growth of the comb in capons. Simultaneous administration of estradiol completely abolishes the effect of the androgen (298).

Although a direct antimetabolite relationship between testosterone and estrone is probably not the only factor operating in the determination of sex character of higher animals, the facts which have been uncovered strongly suggest that it is of importance. The multiplicity of control of function which such an arrangement would give to an individual would be considerably greater than that which could be achieved merely by changing the concentration of one hormone. Thus, if estrone had several biochemical functions, and there is good reason to believe that it has, and if testosterone antagonized only one of these, then an alteration in the concentration of testosterone would affect two changes. The one function of estrone which is dependent on antagonism with testosterone would be inhibited, while the other would be unchecked. If testosterone, *per se*, likewise had an independent function, the same alteration in its concentration which would exert an influence on the functioning of estrone, would, at the same time, bring about another change. A family of controls might thus arise from manipulation of the concentration of one compound.

In a relationship such as that between androgen and estrogen, it is impossible to say which is the metabolite and which the antagonist. From biological data collected in all corners of the field of endocrinology, the estrogen would appear to be the metabolite, but there is almost equal reason for selection of the androgen. Perhaps the truth is that both are metabolites which affect the actions of each other mutually.

One cannot exclude the possibility that the antagonisms of these two kinds of hormones operate through an indirect mechanism. Each may have an independent action not concerned with the other. The morphological and physiological results of each of these actions might chance to be opposites. However, the antimetabolite view of the mat-

ter seems to offer a clearer picture and one which is, thus far, consistent with the experimental findings.

Aside from these speculations, the proof of the validity of the postulate we have been examining will come when it is possible to arrange a simple, *in vitro* system, let us say an enzymic one, in which estrone is required. Probably several of these isolated systems will be needed before the hypothesis can be tested adequately. Testosterone would then be expected to inhibit one of these isolated systems if the postulate is to stand. A beginning has been made in this direction, for estrogens such as diethylstilbestrol have been reported to inhibit the succinic dehydrogenase system of certain tumors (365). The inhibition was observed in suspensions of granules separated from homogenates of tissue. Testosterone or progesterone, in amounts comparable to those of the estrogen, overcame this retardation of the enzyme action. By themselves neither testosterone nor progesterone exerted a measurable effect. Although considerable circumstantial evidence exists to indicate a participation of steroid hormones in the functioning of a succinic dehydrogenase system, the part which they do play is by no means clear. Furthermore, it is not easy to conceive of the entire biological manifestations of these metabolites in terms of such an effect alone. Nevertheless, the demonstration in a cell-free system of the antagonism between diethylstilbestrol and testosterone and between diethylstilbestrol and progesterone bears on the matter we have been discussing.

Antagonism between progesterone and estrogen

The structural similarity of progesterone to the estrogenic hormones may be seen in Figure 16 of Chapter 1. Just as with androgens and estrogens the antagonism between these hormones was found in a series of endocrinological studies which were subsequently taken as evidence that progesterone and estrogen were naturally occurring anti-metabolites. For example, the prolonged administration of estrogen to guinea pigs may result in the appearance of fibroid tumors of the uterus. If progesterone is given along with the hormone, then the tumors are not found (299). A more direct demonstration has been made in young chicks (119). In these animals the injection of estrogen produces a very large growth response in the oviduct. When a maximally effective dose of estradiol or stilbestrol was given, the growth response decreased with increasing concentration of progesterone, simultaneously administered. The data of Figure 2, taken from the work of Hertz and Tullner (119) will illustrate this. The chicks in this experiment received 0.25 milligram of stilbestrol and the progesterone daily for 8 days.

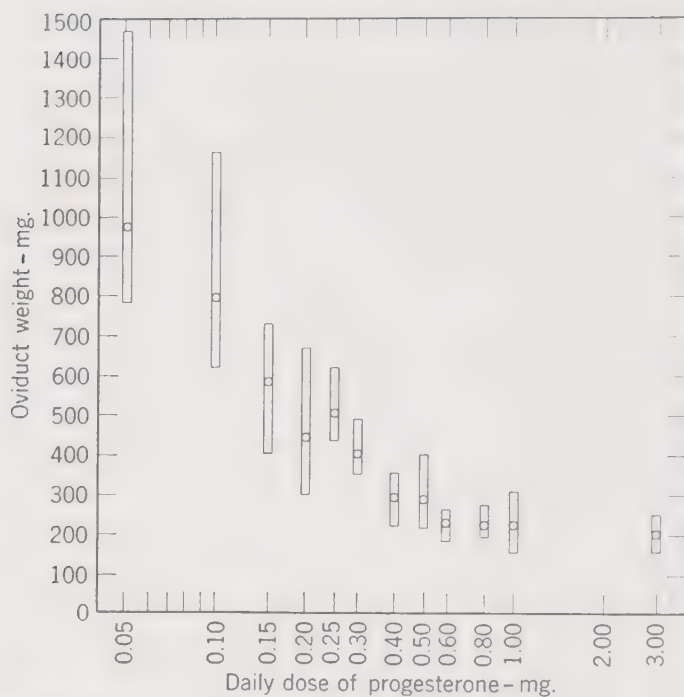


Figure 2. Effect of progesterone on the estrogen-stimulated growth of oviducts of chicks. (Reproduced through the kind permission of Dr. Hertz and the *Ann. N. Y. Acad. Sciences.*)

What was said about the interpretation of the androgen-estrogen antagonism as an example of the antimetabolite phenomenon could be repeated here.

Antagonism between pairs of structurally similar porphyrins

For the bacterium *Hemophilus influenzae*, protoporphyrin or hemin (i.e., iron protoporphyrin) is required for growth, and with this species antagonism can be demonstrated between pairs of naturally occurring compounds of this class (100). For example, if protoporphyrin is used to stimulate growth, then inhibition can be observed if about five times as much hematoporphyrin is added simultaneously to the culture medium. The solubilities of these compounds are so low that it is impractical to determine whether the antagonism is competitive, but the fact of the antagonism is real. Both of the substances involved in the demonstration may occur in the natural environment in which the organism is found. The demonstration of such an antagonism was dependent on the use of a system in which a nutritional requirement for porphyrins could be shown, and such systems are rare. If more were available, the role of these antagonisms in the physiological functioning of the porphyrins might be made clearer.

Antagonism between adenosine and cytidine

Mutant strains of the fungus *Neurospora* are known which require the pyrimidine nucleoside cytidine for growth. For such organisms the purine nucleoside adenosine is toxic, and this action may be reversed competitively by cytidine (300). Both of these compounds are found side by side in living things, and both occur as constituents of nucleic acid. Here, as with the porphyrins, the demonstration of the antagonism depended on the finding of a system in which one of the substances was a required constituent for growth.

Antagonism between desoxyribonucleic acid and ribonucleic acid

Lactobacillus bifidis requires desoxyribonucleic acid as a growth factor when it is cultivated in the absence of Vitamin B₁₂. The nucleic acid can be replaced by one of the desoxyribonucleosides or nucleotides, and presumably the organisms degrade the nucleic acid in the medium to these derivatives before utilization. When growth is supported either by desoxyribonucleic acid or by these derived products, ribonucleic acid causes inhibition of multiplication. The effect is overcome by increasing the concentration of the desoxy compound (463). A similar antagonism has been observed in an isolated enzyme system. Ribonucleic acid has been shown to inhibit the action of desoxyribonuclease on desoxyribonucleic acid. These observations on living cells and on enzymes have suggested that the biological functioning of desoxyribonucleic acid is to some degree regulated by the ribonucleic acid which usually is found along with it in cells.

Antagonism between pairs of amino acids

A large number of cases are known in which a single amino acid, administered in relatively high dosage, is toxic, and in which small amounts of a second amino acid are able to prevent the harmful action of the first. Most of these demonstrations have been made with microorganisms, but a few are known among higher animals. In all of these instances both of the amino acids involved are found normally in the test organism as well as in its food. In many of the examples, but not in all, the antagonism is competitive in kind. With species which require most of the amino acids, and hence lack the ability to convert one into another, the property of one amino acid to overcome the toxic effects of a second one is frequently specific and is not shared by other members of this class of metabolites. With organisms which can synthesize many of the amino acids, or which can convert one of the series into another, the antagonisms are either not found, or, if they are,

they may not be specific for a single substance, i.e., more than one amino acid may be able to overcome the toxic manifestations of a given compound. The individual members of a pair of antagonistic amino acids are usually quite similar in structure, but since nearly all the naturally occurring compounds of this class bear a resemblance this is to be expected. Rather, the specificities which are manifested become the more remarkable.

Let us consider a few examples of this behavior. A pair of closely related antagonistic amino acids has been found in serine and threonine, which differ only in the replacement of one hydrogen atom of the former by a methyl group in the latter. The growth response of several kinds of lactic acid bacteria to serine is conditioned by the amount of threonine simultaneously present (301, 302). More serine is needed for a given amount of growth when the threonine concentration is high than when it is low. Similarly the growth response to small concentrations of threonine is dependent on the amount of serine in the medium. Both threonine and serine are essential to the multiplication of the organisms and each can be shown to antagonize the action of the other. The amounts of these amino acids which are needed to cause such an effect are not beyond those found in the natural environment which the organism might be expected to encounter.

Some of the first examples of antagonism between pairs of similar amino acids were discovered before their possible relationship to the antimetabolite phenomenon was appreciated. One of them will illustrate the problems of specificity mentioned in the opening paragraph of the present section. A mutant strain of *Neurospora* was found which needed lysine for growth. Arginine was observed to inhibit the action of lysine, and no other amino acid was shown to do so to the same extent as did arginine. However, some slight inhibitory action was observed with tryptophane and with norleucine (82). With the parent strain of *Neurospora*, which did not require lysine for growth, no inhibitory effect could be shown with arginine. Both strains of the organism could synthesize all other amino acids. The inhibitory action of arginine for the mutant strain was found with such low amounts of this amino acid that it precluded the recommendation of the use of this organism for quantitative assays for lysine. In other words, the antagonism was evident with concentrations of the two amino acids normally found in natural products. The structural resemblance of arginine or of norleucine to lysine is clear, but that of tryptophane is more remote.

Another example of an antagonism of amino acids in which the lack of specificity is apparent is that involving alanine in the growth of

Streptococcus fecalis. Glycine is antagonistic to alanine in this system, but so also is serine, threonine, or β -alanine (23). Nevertheless, each of these inhibitors of alanine has a close structural resemblance to it and each occurs normally in the environment of the organism.

Considerable question may arise as to how far one should go with the antimetabolite explanation of these amino acid antagonisms. One may even ask whether the hypothesis applies at all to these instances. No simple criterion for deciding this issue seems available. All that can be said is that some of the antagonisms have the general characteristics of the phenomenon as seen with, let us say, a synthetic structural analog of an amino acid, and that metabolite itself. It would be difficult to distinguish between the behavior of a synthetic antimetabolite of an amino acid and the naturally occurring ones. More study of the situation, especially with isolated enzyme systems¹ in which the problem of interconversion of these metabolites has been eliminated, will be required before any authoritative statement of the true explanation can be made. For the present, however, the experimental facts can be understood in terms of the antimetabolite phenomenon.

Possible functioning of an antimetabolite in the natural resistance to infection of bacteria by a virus disease

The natural occurrence of indole, an antimetabolite of tryptophane, is believed to explain why some strains of *Escherichia coli* are resistant to attack by the bacteriophage T-4 (303). When this bacterial virus invades the host cell, the first stage is an attachment to it. In order that this union may occur, tryptophane is specifically required in the medium. This metabolite apparently acts as a link by which the pathogen and the host are joined, and without it no invasion takes place. Tryptophane is normally synthesized by this bacterium so that the virus always finds ready this specific means of infection of the host. The action of tryptophane can be overcome in this process by indole. Indole is structurally related to tryptophane and arises from it in the normal metabolism of *E. coli*. Some strains of the bacteria form indole from tryptophane abundantly. Some strains of this organism also are naturally resistant to the infection by the virus mentioned. The vigorous production of indole conceivably might thus provide a natural protection against the infection. Whether or not this is the mechanism of resistance has not been established without equivocation.

¹ A beginning has been made in the demonstration of competitive antagonism between two similar amino acids in a highly purified enzyme system. Thus the enzymic attack on arginine by arginase has been shown to be inhibited competitively by lysine (366).

Antagonisms between products of one organism and a structurally related metabolite in a second species

Let us turn now from a consideration of antagonisms between pairs of metabolites present in the same individual to an examination of situations in which one species produces a compound which can be seen to be a structural analog of a particular metabolite, and which antagonizes the action of that metabolite in a second type of organism. The harmful product of the first organism does not play the role of metabolite in either of the kinds of living things. Usually it has no recognizable function except, as in the case of the antibiotic products of plants and fungi, it may be part of the defense mechanism of the producing organism. Whether or not these harmful agents are weapons of defense, it is easy to see how some of them might be of value in the struggle for existence.

For these naturally occurring harmful agents, the demonstration of antagonism to a particular metabolite is not so clear as frequently is true with the more orthodox, synthetic antimetabolites. Three factors contribute to this situation. (1) The naturally occurring substances are almost always more different in structure from the related metabolite than are many of the orthodox synthetic antimetabolites. Instead of only one slight change, which is frequently the only difference between metabolite and its synthetic antagonist, the naturally occurring agents have several dissimilarities when compared to the metabolite. (2) The antagonism between the metabolite and the naturally occurring harmful agent is almost always non-competitive. In order to demonstrate it, one must employ minimally toxic concentrations of the harmful substance. If many times this amount are used, no amount of the metabolite will overcome it. (3) High concentrations of the metabolite may be required before any reversal of the action of the antimetabolite can be shown. The amounts needed to nullify the effects of only one toxic dose are frequently many times greater than those which suffice for normal metabolism.

One can see these factors at work in almost every case one examines. Because they do exist, they form the major objections to the view that these naturally occurring harmful relatives of some metabolites actually are antimetabolites. On the other hand, as several of the examples are discussed, it can be seen how closely the behavior of these substances approaches that of orthodox synthetic antimetabolites. Possible reasons can also be seen why the difficulties in demonstrating the

antagonism should exist. For example, one can conceive an advantage to the producing organism in having the weapons it makes, if such they are, not too easily foiled by a mere increase in concentration of metabolite in its victims. Indeed, one sees in work with synthetic antimetabolites a trend to make compounds which are not reversed in their action by the metabolite, and for this same reason. Witness, for example, the attempts to produce sulfonamide drugs which will not be reversed in their action by *p*-aminobenzoic acid. Discussion of a few cases will clarify these principles.

Methoxynaphthoquinone, an antibiotic agent found in garden balsam and its relationship to vitamin K

In a search for antifungal agents in common plants, it was found that one such was present in garden balsam. Isolation and study of it revealed it to be 2-methoxy-1,4-naphthoquinone (210). The resemblance to vitamin K was immediately evident, and it was soon shown (304) that this metabolite, supplied as 2-methyl-1,4-naphthoquinone, would overcome the toxic properties of the antibiotic in some micro-organisms. However, this was not true in all species tested, and even in the ones where it could be shown, the demonstration could only be made over a limited range of concentration. This may have been due in part to the toxicity of methylnaphthoquinone itself.

Iodinin and vitamin K

Chromobacterium iodinum forms a substance which is quite toxic to hemolytic streptococci, and which has been shown to be a dihydroxyphenazine oxide (222) (Figure 3).

The growth-inhibiting action of this compound can be antagonized by small amounts of 2-methylnaphthoquinone, but also by certain naturally occurring anthraquinones. This was the first antagonism between an antibiotic agent and a metabolite to be recognized. The structural resemblance of the agent

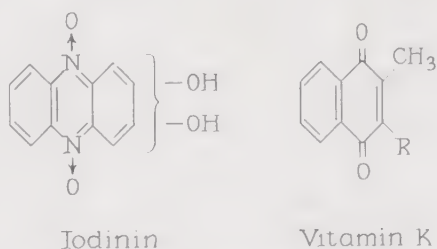
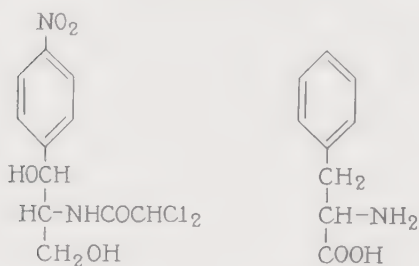


Figure 3. Structures of iodinin and vitamin K.

to the vitamin is not as great as is true for 2-methoxynaphthoquinone, because not only have the carbon atoms in positions 1 and 4 of the naphthalene ring system been replaced by nitrogens, but the alkyl side chains of the vitamin have been changed into a benzenoid ring system in the antibiotic agent.

Chloromycetin and phenylalanine

Certain fungi form a chlorine-containing antibiotic agent which has been found useful in the treatment of infections brought about by Gram-negative bacteria, and by some rickettsiae. This substance, which was named chloromycetin, was found to be of the structure shown in Figure 4 (305). It was recognized to be an analog of phenylalanine (306), although the similarity was somewhat remote in that four structural alterations would be required in order to pass from the metabolite to the antibiotic. Nevertheless, the harmful action of



Chloromycetin Phenylalanine

Figure 4. Structures of chloromycetin and phenylalanine.

chloromycetin towards *Escherichia coli* could be shown to be overcome by phenylalanine, provided that minimally effective doses of the former were used in the tests. Thus, the amount of chloromycetin which would just suppress growth completely (2 gammas per cc.) could be rendered harmless by phenylalanine, but three times this quantity could not be detoxified by the amino acid, even in massive

doses. Of all the other amino acids, only tyrosine and tryptophane were found capable of antagonizing the action of chloromycetin. This relative lack of specificity was probably due to the ability of *E. coli* to interconvert these three amino acids. Such an interconversion has been indicated from the results of diverse experiments. Evidence to support such a view in the present case was found by use of *Lactobacillus casei*. This organism required tyrosine and tryptophane and thus was not able to make them from phenylalanine. This latter amino acid was the only one which overcame the effects of chloromycetin with *L. casei*. A considerably larger amount of the metabolite was needed to antagonize one toxic dose of chloromycetin than was required to promote maximal growth of the organism in the absence of the drug. Thus 20 gammas per cc. of phenylalanine were sufficient for growth, but about 200 gammas were needed to interfere with the chloromycetin.

An effort was made to determine whether chloromycetin could properly be regarded as an antiphenylalanine by studying the nature of the antagonism between that amino acid and each of the analogs between it and the antibiotic. For this study a mutant strain of *E. coli* was used which required phenylalanine for growth. The reason for this selec-

tion will appear shortly. When just one structural change was made in the metabolite, four analogs were achieved. Of these dichloroacetylphenylalanine was not toxic, but, rather, showed slight metabolite potency (see Table 3). *p*-Nitrophenylalanine and hydroxyaminophenylpropane were relatively inert. The fourth analog, viz., β -hydroxyphenylalanine, was a rather weak but competitive antagonist, as had been shown earlier for other bacteria (96). When two or three of

Table 3

Toxicity for a phenylalanine-requiring strain of *E. coli* of compounds intermediate in structure between chloromycetin and phenylalanine, and the ability of phenylalanine to overcome it

(Data in this table are reproduced from literature (306).)

No.	Compound	Toxicity *				Type of Antagonism
		With 5 gammas Ph. A.	With 10 gammas Ph. A.	With 20 gammas Ph. A.	With 200 gammas Ph. A.	
1	Dichloroacetylphenylalanine	Phenylalanine activity			
2	1-Hydroxy-2-amino-3-phenylpropane	Nothing at 4.0			
3	<i>p</i> -Nitrophenylalanine	Nothing at 1.0	Nothing at 1.0		
4	β -Hydroxyphenylalanine	1.0 Never complete	6.8	13.0		Competitive †
5	1-Hydroxy-2-(dichloroacetyl-amino)-3-phenylpropane	Nothing at 0.6			
6	β -Hydroxy- <i>p</i> -nitrophenylalanine	0.8		1.2	Nothing at 2.0	Non-competitive
7	Dichloroacetyl- <i>p</i> -nitrophenylalanine	6.0	8.0			Non-competitive
8	1-Phenyl-2-amino-1,3-propanediol	Nothing at 2.0		Nothing at 6.0	
9	N-dichloroacetyl- β -hydroxyphenylalanine	Nothing at 3.0			
10	1-Phenyl-2-dichloroacetylamido-1,3-propanediol	0.05	0.10	0.20	0.10	Competitive below 0.2 mg., then irreversible
11	N-dichloroacetyl- β -hydroxy- <i>p</i> -nitrophenylalanine	0.5	1.0	1.5		Competitive below 1.5 mg.
12	1-(<i>p</i> -nitrophenyl)-2-amino-1,3-propanediol	0.2		0.6	0.6	Competitive below 0.6 mg., then irreversible
13	Chloromycetin	0.0018	0.0027		0.0031	Non-competitive

* Expressed as the amount (in milligrams per cubic centimeter of culture) required to cause half maximal inhibition of growth in the presence of graded concentrations of phenylalanine.

† The competitive nature of the antagonism is obscured by some phenylalanine activity which makes itself felt with low concentrations of the metabolite.

these modifications were made simultaneously, the compounds obtained either were inert or were antagonized with some difficulty by phenylalanine. Several of these analogs were competitive antagonists when tested at low concentrations but rapidly assumed a completely irreversible toxicity as the concentration passed a critical value. This was true, for example, with compounds 10, 11, and 12 of Table 3. The emergence of irreversible toxicity, or of toxicity difficult to antagonize with the metabolite, was associated with the introduction of nitro- and dichloroacetyl groups into the molecule. This can be appreciated readily by comparison of the behavior of compounds 4, 6, and 11 or of compounds 8, 10, and 13.

The advantage of using a phenylalanine-requiring organism for such a study was readily seen when the same series of compounds was tested with the parent strain of *E. coli* which did not need the metabolite nutritionally. The relative toxicities of the various substances were about the same as those for the phenylalanine-dependent strain grown in the presence of 20 gammas of this amino acid per cubic centimeter. The main exception was with β -hydroxyphenylalanine, which for the parent strain was more injurious in the absence of added phenylalanine. A study of the values in Table 3 will show that this meant that for the parent strain no antagonism by the metabolite could be shown for compounds 10, 11, and 12 because even to the mutant in the presence of 20 gammas per cubic centimeter of phenylalanine they were irreversibly poisonous. These substances apparently acted in a *reversible* fashion only on species with nutritional needs for phenylalanine, and then only at relatively low concentration. Indeed trials with *L. casei* which also requires this amino acid supported this idea.

From these facts it was concluded that loss of competitive character in the antagonism was probably related to progressive alteration of the structure, and also to the introduction of nitro- and dichloroacetyl groups. This second influence seemed to contribute to a type of toxicity which was not influenced by phenylalanine. Possibly this was due to irreversible binding of compounds with such groups to the site of action. Mere increase in concentration of phenylalanine could not then displace such a bound antagonist. No explanation could be offered for the marked and irregular variation in potency within the series of compounds. If high potency depended on binding by nitro- and dichloroacetyl groups, one would expect a substance such as N-dichloroacetyl- β -hydroxy-*p*-nitrophenylalanine to be almost as powerful as chloromycetin, but it was not. Rather, it seemed that high activity, as in chloromycetin, arose when competitive antagonism was completely lacking. From the demonstration of structural similarity

and biological antagonism, it was suggested that one aspect of the antibacterial action of chloromycetin was the following. The compound acted as an inhibitor of a vital process in which phenylalanine was transformed into a product essential in bacterial cell multiplication. The character of chloromycetin was such that in this inhibition phenylalanine was able only partially to antagonize the action of the drug, possibly for the reasons just indicated.

Regardless of opinions about them, the facts are: (1) Small doses of chloromycetin can be antagonized effectively by phenylalanine. (2) The effect is non-competitive and disappears completely as the concentration of chloromycetin is raised. (3) In series of compounds stretching progressively from the metabolite to the antibiotic, the competitive character of the antagonism is lost as the similarity to the metabolite decreases.

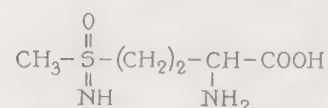
Reversal of the antibacterial action of penicillin with yeast extract

It is said that the toxic effect of penicillin against strains of the diphtheria bacillus is overcome by a peptide-like material extracted from yeast (307). This extract contained an essential but unidentified growth factor for the organisms used. Since properties of this growth factor suggested that it was related to a peptide, and since penicillin has structural features similar to those which some investigators postulate to exist in proteins, it was suggested that the antagonist to penicillin action was a natural metabolite against which the effects of the antibiotic were exerted. The secret of success in this demonstration of antagonism seemed to lie in the use of an organism which showed a nutritional requirement for the metabolite. Most of the common bacteria do not need an exogenous source of this growth factor which the diphtheria bacillus requires. If this view of penicillin action is substantiated, we would see once again that with a naturally occurring antimetabolite, just as with phenylpantothenone or with glucoascorbic acid (cf. Chapter 2), that the ability to demonstrate antagonism with the metabolite is correlated with the nutritional requirement of the organism for the latter.

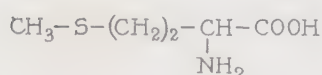
Methionine sulfoximine, the causative agent of canine hysteria, and its antagonism by methionine

The following example is included in this chapter because it involves an antimetabolite which is the causative agent of a disease of dogs. Although this compound is found in foods, it is not a natural product but rather is formed in the processes of manufacturing these foods. When dogs are fed a ration containing much wheat flour, they develop

convulsive seizures, known as running fits or canine hysteria. Unmilled wheat is not harmful, whereas the manufactured products from it are. This difference was traced to the use of nitrogen trichloride with which flour was usually treated to improve its baking qualities (445). Nitro-



Methionine sulfoximine



Methionine

Figure 5. Methionine sulfoximine and methionine.

gen trichloride reacted with the wheat proteins to form the toxic agent. This latter was isolated (after hydrolysis of the proteins) and identified as methionine sulfoximine (see Figure 5) (442, 443, 446). The convulsant action in dogs or in rabbits of this analog of methionine could be overcome by that amino acid (447). Furthermore, the substance was quite inhibitory of growth of some bacteria, such as *Leuconostoc mesenteroides*, and, for this organism also, methionine would overcome the harmful effects (444, 448). The sulfoximine was believed to produce a methionine deficiency of nerve tissue, and thus to be responsible for the disease (447).

Naturally occurring antimetabolites as etiologic agents of disease

Some diseases of higher animals and of economic plants seem to be caused by toxic agents elaborated by other species, and these harmful substances are related in structure to certain metabolites. In a sense, this is just a special case of the situation we were just examining, because the inhibition of the growth of *E. coli* by chloromycetin is certainly a disease of that organism caused by a harmful fungal product. But, since we usually are not concerned about diseases of bacteria and are interested in maladies of higher animals and plants, the separate discussion of these examples seems justifiable.

Enough has probably been said about 3,3'-methylenebis-(4-hydroxycoumarin) in previous chapters to show its similarity to vitamin K. The fact that it calls forth in animals the signs of vitamin K deficiency, and that over a limited range of concentration, large doses of the vitamin will nullify its effects has also been discussed. The relationship of the two substances is not competitive, and, furthermore, doses of vitamin K about 1,000 times those required nutritionally are needed to antagonize the action of the coumarin. This toxic agent was discovered because when sweet-clover hay spoils as a result of exposure to moisture it acquires a toxicity such that when it is eaten by farm animals a hemorrhagic disease is produced which can be quite fatal. During the spoiling the ordinary coumarin of the sweet clover is apparently converted to this toxic agent by microbial action. This struc-

tural relative of vitamin K is the etiological agent of the hemorrhagic disease. Just as with chloromycetin or with iodinin, the structural similarity of the metabolite to the antimetabolite is not close, and in this fact may be one reason why the antagonism is not competitive.

Another example suffers from lack of knowledge of the structure of the metabolite but may be worthy of note. Tomato plants are subject, especially when grown on poor soil, to infection by *Fusarium lycopersici*. The disease so produced is characterized by the wilting and curling of the leaves of the plant. When the responsible fungus is grown *in vitro*, it forms a toxin which causes excised tomato leaves to wilt

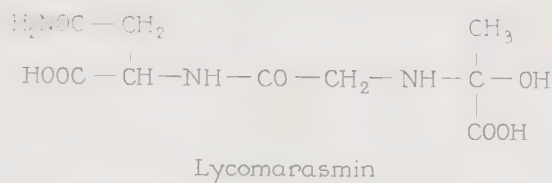


Figure 6. Structure proposed for the tomato-leaf-wilting toxin of *Fusarium lycopersici*.

much as they do on diseased plants. The toxin has been isolated (310) and shown to be a peptide derivative of asparagine, glycine, and α -hydroxyalanine (311). Its structure is probably that shown in Figure 6. The action of this toxin on excised tomato leaves can be prevented by small amounts of concentrates of the growth factor strepogenin (312). This antagonism is non-competitive and is seen only with small doses of the toxin. Although the structure of strepogenin is not known, fair evidence exists to indicate that it is a peptide containing glutamic acid and glycine. A synthetic peptide, serylglycylglutamic acid, possesses strepogenin activity as a bacterial growth factor and is able to antagonize the action of the leaf-wilting toxin on tomato leaves. Indeed, it was the recognition of the antagonism between the toxin and the growth factor and the knowledge of the structure of the toxin which led to the synthesis and trial of serylglycylglutamic acid. When it is recalled that serine is β -hydroxyalanine, the structural similarity to the toxin can be recognized. Although serylglycylglutamic acid had a strepogenin activity, this was quantitatively too small to suggest that the naturally occurring growth factor was identical with the synthetic peptide. When the chemical constitution of the natural growth factor is known, there will be better grounds on which to discuss whether the toxin is a naturally occurring antimetabolite. In the meantime, one must remember that all antagonisms in nature are not of the antimetabolite variety.

CHAPTER 6

Selectivity of action of antimetabolites

The existence of selectivity

In common with many other substances, antimetabolites usually affect some organisms, or tissues of a single individual, much more than others. This selectivity of action has many aspects, and it will be the purpose of this chapter to investigate a few of them. Most of these aspects are so poorly understood that practically nothing of an exact character can be said of them. Therefore, we can make only a beginning.

Although a single antimetabolite may exert its effects over the entire range of living things, within each class of organisms individual species are found which are quite insusceptible. This can be seen well from the experiences with pyriithiamine. Thus, although higher animals, invertebrates, fungi, bacteria, and even enzyme systems are inhibited in their activities by this analog of thiamine, many species of bacteria, fungi, and plants are known which are not at all affected by it. The generic difference between a resistant and a susceptible form may be very slight, so that an explanation based on gross and fundamental morphological and physiological deviation does not seem adequate. This inadequacy appears even more true when we reflect on the similar susceptibility of widely separated species.

Another aspect of selectivity of action can be seen when several analogs of the same metabolite are examined. The range of species susceptibility to one analog may not be the same as for another. A good example of such behavior is found with the antivitamin K. Thus, 3,3'-methylenebis-(4-hydroxycoumarin) affects animals and is relatively non-toxic to most microorganisms (420). A different type of

structural relative of the vitamin, namely, 2,3-dichloronaphthoquinone, is practically harmless to animals but exceedingly poisonous to fungi and to certain other microorganisms (209). Similarly, the derivatives of sulfanilamide, which are analogs of *p*-aminobenzoic acid, affect adversely a great array of microbial species and leave the higher animals relatively unharmed, while atoxyl, the arsonic acid analog of *p*-aminobenzoic acid, is poisonous to both classes of organism, even though the higher animals usually are not quite so susceptible.

The importance of selectivity

The understanding of why selectivity exists is important, not only to satisfy curiosity about natural phenomena but also from a practical standpoint. An insight into the reasons for selectivity, and a knowledge of how to control it, are fundamental to any therapeutic applications of antimetabolites, aside from those based solely on chance.¹ Likewise, the unequivocal use of antimetabolites in the study of biochemical processes, especially those in living cells, must depend to some extent on such knowledge. Let us, therefore, inquire into the problem to the limits of present information and hypothesis.

Multiplicity of aspects of the problem

So far as can be determined, there is no one explanation of selectivity. Rather, a given agent may owe its ability to affect one organism or tissue, and to leave others unharmed, to a metabolic difference in the two, whereas a second agent may depend on physical factors such as relative solubility for its selective effects (449, 450). In a living organism the selectivity observed may be the resultant of several of these aspects of the situation, but one of them may be the predominating factor. Because of this multiplicity of reasons for selectivity, the explanation of any given case must weigh the several aspects of the problem.

Two distinct divisions of the question of selectivity seem evident. One deals with the reasons why one single compound affects species A but not species B when each type of living thing is examined in the absence of others. The other is concerned with the reasons why a compound harms species A but not species B when the two are grown together. In the present chapter, data will be cited to indicate that the second of these aspects is much more complex than the first. Obviously, however, it is the second one which is directly concerned in the chemotherapy of infectious diseases. Although a substance may

¹ This conclusion is not without foundation as witnessed by the growing number of investigators who subscribe to it (see, for example, 314, 449, 450).

harm species A and not influence species B when each is examined alone, when the two are grown together neither may be affected by the substance. One reason for this is that species B can destroy the compound, thus detoxifying it not only for itself but also for species A. Several examples of such behavior are known, and some will be discussed in the next sections. Other reasons aside from destruction of the compound have likewise been discerned. However, as a beginning in the study of selective toxicity, the simplest question to answer is why individual species cultivated by themselves vary in susceptibility to a specified substance. With some understanding of this, the second division of the problem may be more readily approached.

Correlation of selectivity with nutritional requirement for the related metabolite

This correlation, which deals with isolated single species, was one of the first facts about selectivity of action of antimetabolites to be discovered because it could be seen clearly from the testing of antivitamin on a variety of species. It is illustrated by the data of Table 3 in Chapter 1, and of Tables 1, 2, and 3 of Chapter 2. Thus pyrithiamine inhibits the growth only of those microorganisms which require thiamine or one of its two constituent parts as growth factors. The inhibition seems to be more powerful, i.e., less analog is needed to cause it, if the intact vitamin is demanded than if one of the halves of the molecule suffices (5). Similarly, pantooyltaurine affects those microorganisms which will not grow without pantothenic acid and does not influence the multiplication of others (171). However, as already discussed, the correlation of action of the antimetabolite with nutritional need for the related metabolite is not always found. Modifications have also been indicated.

Why this correlation exists has not been explained satisfactorily. It may be that the uptake of the nutritionally required metabolite is not a passive process of diffusion through the cell membrane but rather is an active metabolic reaction in which the antimetabolite can participate as well as its physiologic relative. Just as an organism can phosphorylate desoxypyridoxine before it functions as an antagonist to pyridoxal phosphate (146), so other antimetabolites may undergo a relatively non-exacting metabolic step before passing into the cell. This phosphorylation or other change is believed by some investigators to be essential to the uptake. Metabolic reaction systems are known to vary widely in their ability to handle foreign substances. Once thus transported into the cell, the antimetabolite can then exert its antagonistic action to the metabolite. If, however, the metabolite is made

within the cell so that there is no special mechanism provided for its uptake from the environment, the antimetabolite would be unable to enter. Only circumstantial evidence, and no direct proof, of this idea is available. Until such proof is had, one must be content to recognize and to use this correlation without understanding it adequately.

Selectivity based on lack of a metabolic function in some species

When an organism lacks a function involving a given metabolite, analogs of that metabolite may be unable to affect it, although they are quite harmful to those forms which possess the function, and depend on it. The resistant species frequently make good their lack of function by relying on the environment to supply them with what, in the susceptible forms, is made from the metabolite within the cell. The lack of function can thus be found experimentally by determination of nutritional needs. Nevertheless, the correlation with nutritional requirements is quite different from that described in the preceding section, because here, in contrast to the situation there, the resistant organisms are the ones which have the special growth factor requirement. Let us follow the genesis of this idea because it seems to be of importance to chemotherapy.

When the structure of folic acid (pteroylglutamic acid) became known (281) and *p*-aminobenzoic acid was seen to be a part of it, the functioning of sulfanilamide and its derivatives became much clearer. These drugs competed with *p*-aminobenzoic acid acting as a substrate in a series of reactions of which folic acid was the product. Lampen and Jones (245), and subsequently others (246, 313, 438), supplied evidence for this by showing that in selected species of bacteria, folic acid would antagonize the action of sulfanilamide non-competitively, whereas *p*-aminobenzoic acid would do so competitively. By supplying the product of the inhibited reaction, the inhibition was circumvented. Data illustrative of this have been given in Table 1 of Chapter 3. They also observed that those few species of bacteria which required folic acid were remarkably resistant to sulfonamide drugs. This fact can be seen from their experiments as well as from the more recent and extensive results of Aubagen, which are shown in Table 1 (246). The reason why the sulfonamide drugs could inhibit the growth of many microorganisms, and still not injure an animal, then seemed much clearer (286, 450) because animals in general require folic acid as a dietary essential, and thus lack the function which the sulfonamides inhibit. Most bacteria which have been examined make their own folic acid. The key to one aspect of the selectivity of the sulfon-

Table 1

Correlation of sulfanilamide resistance with folic acid requirement and the ability of folic acid to antagonize the action of the drug

(Reproduced with the kind permission of Dr. Auhagen and the *Z. physiol. Chem.*)

Organism	Growth without Sulfanilamide		Growth with Sulfanilamide	
	Without folic	With folic	Without folic	With folic
<i>Escherichia coli</i>	+	+	—	—
<i>Bacillus subtilis</i>	+	+	—	—
<i>Staphylococcus aureus</i>	+	+	—	—
<i>Streptobacterium plantarum</i>	+	+	—	—
<i>Thermobacterium helveticus</i>	+	+	—	+
<i>Enterococcus</i> W12	+	+	—	+
<i>Enterococcus</i> W13	+	+	—	+
<i>Enterococcus</i> W14	+	+	—	+
<i>Enterococcus</i> W15	+	+	—	+
<i>Enterococcus</i> W16	+	+	—	+
<i>Enterococcus</i> W17	+	+	—	+
<i>Enterococcus</i> W18	+	+	—	+
<i>Streptococcus viridans</i> E	+	+	—	+
<i>Bacterium</i> AM.B.	+	+	—	+
<i>Streptococcus fecalis</i> R	—	+	—	+
<i>Lactobacillus casei</i>	—	+	—	+

amide drugs thus appeared to be the possession or lack of the ability to synthesize folic acid.

In order to test this hypothesis further, it was applied to a new pair of metabolites, one of which was precursor of the other (314, 450). Pimelic acid is apparently a biological starting point for biotin just as

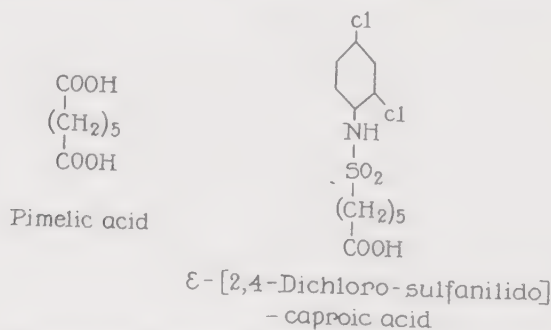


Figure 1. Structures of pimelic acid and of an antimetabolite of it.

p-aminobenzoic acid is of folic acid. Analogs of pimelic acid were, therefore, made in the expectation that they would affect those species which synthesized biotin but would not harm those which did not. The most active compound tested was the one shown in Figure 1, which

is related to pimelic acid in that the one carboxyl group of the metabolite has been replaced by a dichlorosulfanilide radical. It inhibited the growth of several species of bacteria which did not require biotin but was without effect on those which did. Mice, as well as these other biotin-requiring forms, were resistant to it, and it was pointed out that mice have a nutritional need for biotin. The growth-inhibiting action of the analog was overcome by pimelic acid competitively, and non-competitively by biotin. Here too, then, one major aspect of the selectivity seemed to be the possession of, or lack of, a nutritional need for biotin. Resistance was correlated with the need for this vitamin. Data to illustrate this point, taken from a published paper (314), are shown in Table 2. A requirement for the metabolite to which the harmful

Table 2

Correlation of toxicity of ϵ -[2,4-dichlorosulfanilido]-caproic acid with nutritional needs for biotin

Organism	Analog Needed for Half Maximal Inhibition, gammas per cc.	Nutritional Requirement for Biotin
<i>Bacillus tenuis</i>	58 *	Not required but stimulatory under certain conditions
<i>Acetobacter suboxydans</i>	350	Not required but somewhat stimulatory
<i>Mycobacterium tuberculosis</i> H37Rv	20 †	Not required
<i>Escherichia coli</i>	No effect at 1,000	Not required
<i>Escherichia coli</i> biotin-less	No effect at 1,000	Required
<i>Proteus</i> strain 4	No effect at 1,000	Required
<i>Staphylococcus aureus</i>	No effect at 300	Required
<i>Lactobacillus casei</i>	No effect at 500	Required
<i>Lactobacillus arabinosus</i>	No effect at 500	Required
<i>Leuconostoc mesenteroides</i>	No effect at 500	Required
Hemolytic streptococcus H69D	No effect at 500	Required
<i>Saccharomyces cerevisiae</i>	No effect at 1,000	Required

* When cultured in test tubes a value of 20 was uniformly found.

† Incubation time 4 days.

agent is related is not the point in question but rather the need for a second metabolite for which the former is a precursor is the deciding feature. Resistance is related to a deficiency in the function of forming biotin, or folic acid, as the case may be. An intensive study of antimetabolites related to precursors of important metabolites seems to be indicated.

Although the foregoing aspect of the selectivity of action appears to be important, it is not the only one in the examples cited. This be-

comes evident from the fact that all sulfonamide-resistant bacteria do not require folic acid. Strains of streptococci and of pneumococci are known which are not inhibited at all in growth by sulfonamide drugs, and yet do not differ from the parent-susceptible strains in their ability to synthesize folic acid. This same discrepancy appeared with one species in the study of analogs of pimelic acid. For *Escherichia coli*, the dichlorosulfanilide related to pimelic acid was harmless even though this organism synthesized biotin. Indeed, the presence of the analog did not even reduce the amount of biotin which was formed during growth. Some additional reason for the selective action of the antimetabolites must be involved in these cases if the thesis of this section is to be retained. One such additional reason is available to help in the understanding of the resistance of some bacteria to sulfonamide drugs. This is that they form an amount of *p*-aminobenzoic acid large enough to antagonize the action of high concentrations of the drugs (315). However, only a few of the cases can be thus explained and not all of them.

Although the analog of pimelic acid had been successful as an inhibitor of growth of biotin-independent species, it had been tested against isolated, pure cultures of organisms. The next important question was whether it would show a selective action in mixed cultures. Quite probably it would not. The test with mixed cultures must necessarily be conducted in media containing biotin, and this would render the analog harmless even for susceptible strains. Accordingly, when attempts were made to retard the multiplication of tubercle bacilli (a susceptible species) in mice, they failed² (451). Since the mice contained biotin of dietary origin, a plausible explanation for this result could thus be offered. Nevertheless, it was not proved beyond equivocation that this is the true explanation.

Reconsideration of the known facts about sulfanilamide action may possibly be used as the basis of a working hypothesis which would allow attack on the problem of how to deal with one organism in the presence of another. Sulfanilamide will harm bacterial species by retarding the synthesis of folic acid, as has already been discussed. In a very limited number of these species, folic acid will nullify the growth inhibition in a non-competitive fashion. When these latter organisms invade animal tissues, sulfanilamide has no chemotherapeutic effect against them. One reason may be the presence of folic acid in con-

² It is futile to attempt to overcome this difficulty by use of larger doses of the antimetabolite because, as was shown earlier, the antagonism exerted by biotin is non-competitive. In other words, an amount of biotin which will antagonize a small concentration of the antimetabolite will likewise controvert a large amount.

siderable concentration in such tissues. However, for the pathogenic species which are controllable in animals by sulfanilamide (pneumococci, streptococci, etc.), folic acid has no outstanding ability to antagonize the drug. Why is this so?

One working hypothesis is that *p*-aminobenzoic acid is a biological precursor not only of folic acid but also of other essential metabolites concerned in cell division.³ The simultaneous presence of these metabolites, in addition to folic acid, would be needed before the toxicity of the drug could be circumvented.⁴ Perhaps in some poorly understood way not even a mixture of all of them would be able to nullify the effect of the antimetabolite, and this would then be why, with pneumococci and streptococci, growth can be inhibited even in a living animal. This idea can be tested in the following way. Another metabolic precursor (unrelated to *p*-aminobenzoic acid) should be sought which is used in the biosynthesis of *two or more* essential participants in cell division. A suitable analog of such a precursor should then be constructed, and this should prove to duplicate the selective action which sulfanilamide shows in mixed cultures. This has been attempted, and some evidence compatible with the idea just mentioned has been found (452). The selectively toxic analog so produced actually showed the toxicity for some species and the harmlessness to others which had been predicted. In addition, the two vitamins which normally arise from the precursor were not able to antagonize its growth-inhibiting effects.

The precursors of most vital metabolites are not known, so that to choose one which is used to form two of them is very difficult. Any choice must involve some assumptions. However, 1,2-dimethyl-4,5-diaminobenzene is found in two important vitamins, viz., riboflavin and vitamin B₁₂, and conceivably could be a metabolic precursor of both of them. In riboflavin, this molecule has been condensed with a ribityl group and with alloxan, and thus incorporated into an isoalloxazine ring system. In vitamin B₁₂ it is built into a benzimidazole riboside (453, 454). Experimental evidence has been found which is compatible with the idea that this dimethyldiaminobenzene probably is the specified precursor of both vitamins. It had some activity as a

³ Those which have been mentioned previously, namely the purines and amino acids, are not the ones intended here because considerable evidence exists to show that they are concerned with the action of folic acid and thus only indirectly with *p*-aminobenzoic acid. Compare Chapter 9.

⁴ This point of view has been presented elsewhere in this book and need not be repeated here. It should also be unnecessary to reiterate that divergent opinions about the matter have been championed.

substitute for riboflavin, and also for vitamin B₁₂ (452) in promoting growth of microorganisms. The potencies observed were only about 10⁻⁶ that of the intact vitamins, but they appeared to be real. When the ribityl group was attached, the potency as riboflavin increased, whereas that as vitamin B₁₂ disappeared. The substance in question, namely, 1-ribitylamino-2-amino-4,5-dimethylbenzene, is closer to riboflavin and more distant from vitamin B₁₂. More direct evidence for participation of the 1,2-dimethyl-4,5-diaminobenzene in the biosynthesis of vitamin B₁₂ was that the addition of this compound to growing bacterial cultures increased the amount of this vitamin formed without simultaneously affecting growth (490).

A suitable analog of dimethyldiaminobenzene would be expected to harm those species which synthesized riboflavin or vitamin B₁₂, and particularly those which make both of these. It should be relatively inert towards those which have a nutritional requirement for both vitamins. This would be the implication of the idea discussed for the pimelic acid analog. In addition, if the working hypothesis just outlined were correct, then riboflavin alone should not overcome this toxicity, and neither should vitamin B₁₂. In fact, even a mixture of the two should be unable to erase the deleterious effect on growth. An analog of dimethyldiaminobenzene which would compete with it was therefore constructed and actually showed the selectivity desired. This substance was 1,2-dichloro-4,5-diaminobenzene, which is related to the postulated precursor in that chlorine atoms have replaced the methyl groups on the benzene ring.

The data in Table 3 will show that this analog was toxic to those bacteria, fungi, and algae which synthesize their own supplies of either riboflavin or vitamin B₁₂, or both. It was not harmful to those which could not synthesize either but required them preformed in the medium. Mice are among these latter forms, and, in accord with the working hypothesis, they tolerated large doses of the analog. Thus 16 mice were given 0.5 per cent of their ration as the dichlorodiaminobenzene, and in addition were injected daily with 6 milligrams of the substance, and yet they gained weight and thrived for a period of 4 weeks. Riboflavin plus vitamin B₁₂ was incapable of overcoming the toxicity of this analog for all the susceptible species tested. This might be a deterrent to the conclusion that the dichlorodiaminobenzene was retarding the synthesis of these vital metabolites, but it was the result which was expected if the working hypothesis, deduced from the action of the sulfonamides, was to stand. The growth-inhibiting properties were, however, antagonized, and in a competitive fashion, by 1,2-dimethyl-4,5-diaminobenzene, the postulated metabolic precursor.

Table 3

Toxicity of 1,2-dichloro-4,5-diaminobenzene correlated with nutritional requirements for riboflavin and vitamin B₁₂. (Toxicity is expressed as amount of the dihydrochloride which caused half maximal inhibition of growth in a synthetic medium containing all known nutrients.)

Organism	Amount of Analog, gammas per cc.	Riboflavin Requirement	B ₁₂ Requirement
<i>Lactobacillus brevis</i>	6	None	None
<i>Lactobacillus arabinosus</i>	10	None	None
<i>Saccharomyces cerevisiae</i>	6	None	None
<i>Staphylococcus aureus</i>	6	None	None
<i>Streptococcus fecalis</i> R	40	None	None
<i>Bacillus tenuis</i>	20	None	None
<i>Chlorella vulgaris</i>	10	None	None
<i>Ophiostoma multiannulatum</i> mutant strain 1671	20	None	None
<i>Leuconostoc mesenteroides</i>	4	None	None
<i>Xanthomonas pelargonii</i>	1	None	None
<i>Corynebacterium fascians</i>	1	None	None
<i>Corynebacterium michiganense</i>	0.6	None	None
<i>Pseudomonas angulata</i>	100	None	None
<i>Pseudomonas tabaci</i>	30	None	None
<i>Agrobacterium tumefaciens</i>	60	None	None
<i>Escherichia coli</i>	200	None	None
<i>Proteus</i> strain 4	100	None	None
<i>Shigella sonnei</i>	200	None	None
<i>Salmonella typhimurium</i>	100	None	None
<i>Lactobacillus casei</i>	140	Required	None
Hemolytic streptococcus H69D	180	Required	None
<i>Euglena gracilis</i>	100	None	Required
<i>Lactobacillus leichmannii</i> strain 313	No effect at 300 *	Required	Required
<i>Lactobacillus bifidis</i> strain 4963	No effect at 300	Required	Required
<i>Lactobacillus lactis</i> (Dorner) strain 8000	No effect at 300	Required	Required

* This was the largest concentration which could be dissolved in test media.

sor.⁵ Because riboflavin plus vitamin B₁₂ was unable to nullify the toxicity of this poisonous substance, one presumed cause for failure to act selectively on one species in a mixture of organisms (the cause discussed for the pimelic acid analog) was removed. Trials of whether it would so act have been only preliminary but have seemed to succeed

⁵ The amount of dimethyldiaminobenzene needed for demonstration of antagonism to the dichlorodiaminobenzene was larger than might be expected. About 1 gamma of it per cubic centimeter was the minimally effective concentration. Because both riboflavin and vitamin B₁₂ are maximally active at less than 10 per cent of this concentration, one may ask why less dimethyldiaminobenzene did not suffice. Furthermore, not only the dimethyldiaminobenzene but, to a lesser extent, *ortho*-phenylene-

(455). Thus, it has been possible to inhibit the growth of susceptible organisms (those which make riboflavin and vitamin B₁₂) in the presence of resistant species.

Because of the failure of riboflavin plus vitamin B₁₂ to nullify the effects of the dichlorodiaminobenzene, one may question whether the toxicity of this substance is, in fact, due to its interference in the synthesis of those vitamins. Evidence compatible with the idea that it is has been found. This is that when microorganisms such as *Bacillus megatherium* are grown in the presence of non-toxic amounts of the dichlorodiaminobenzene, the quantities of vitamin B₁₂ and of riboflavin which are synthesized are reduced (490).

If the idea that selectively and irreversibly active compounds can be made by changing the structure of a precursor shared by two or more vitamins is correct, then it is also clear that indiscriminate choice of the precursor involved will not succeed. For example, glutamic acid is held to be a precursor of several essential metabolites, two of which are glutamine and glutathione. Antimetabolites of glutamic acid have not been found to show selective action on organisms which synthesize both glutamine and glutathione; neither have these antimetabolites been poisons irreversible by glutamine plus glutathione. However, in the case both of sulfanilamide and of 1,2-dichloro-4,5-diaminobenzene, the products derived from the analogous precursors (folic acid, riboflavin, vitamin B₁₂) are believed⁶ to be directly associated with the processes of cell division. It is possible that the interference with cell division which sulfanilamide and dichlorodiaminobenzene bring about, in conjunction with their selective properties, is related to this.

The illustration involving 1,2-dichloro-4,5-diaminobenzene may add some evidence in favor of the postulate that selectively acting compounds can be made by alteration of the structure of a precursor of an essential substance. Knowledge of the nutritional requirements of different species allows prediction of which will be harmed, and which will not. However, in assessing the validity of the idea which led to these results, the assumptions involved must not be forgotten. Especially is this so for the postulate about how to proceed with agents for mixed cultures. Evidence in support of these assumptions was de-

diamine showed some ability to overcome the effect of the dichloro analog. There is no reason to believe that phenylenediamine is a precursor of any of the vitamins. Therefore, these two findings may speak against the acceptance of the dimethyldiaminobenzene as a bonafide metabolic precursor of riboflavin and vitamin B₁₂.

⁶ Evidence compatible with this belief has been presented in a number of investigations in several laboratories on the functioning of these vitamins and will not be discussed in detail here. The evidence is suggestive rather than conclusive.

duced, but complete proof was lacking. The chief justification for discussion of the working hypothesis is that it has led directly to a small but considerable amount of experimental verification of its predictions.

Selectivity based on variation among organisms of ability to demonstrate antagonism with the metabolite

If an organism which would otherwise be susceptible to the effects of an antimetabolite should form large quantities of the related metabolite, or of antagonistic substances other than this one, it might be able to combat successfully the toxic action to which other species succumb. This is apparently the case with a few strains of bacteria, discussed in the early part of the preceding section, which form enough *p*-aminobenzoic acid to account for their ability to withstand sulfonamide drugs. This is probably a situation to be reckoned with when one attempts to make selectively active agents, but at present it does not seem to be amenable to control except by the formation of irreversibly acting antimetabolites. It may explain why an isolated species is resistant to a compound and a second one is not. However, if the two organisms are grown side by side the excess metabolite formed by the one will also antagonize the drug and allow growth of the other species. Selectivity would thus depend on trials with isolated organisms and would not be of practical significance.

However, if the metabolite overcame the effects of the analog in one type of living thing and was unable to do so in a second species, then the excess antagonistic metabolite would protect the first but not the second. Such a selectively acting system may be demonstrated experimentally with certain derivatives of pantothenic acid and of folic acid. *Saccharomyces cerevisiae* and *Lactobacillus casei* are both susceptible to inhibition by phenylpantothenone. Pantothenic acid will antagonize this effect in the latter but not in the former species. Therefore, if an excess of that vitamin is present in the medium along with large amounts of phenylpantothenone, *Lactobacillus* will grow and *Saccharomyces* will not. This was readily seen when the two organisms were inoculated into a medium adequate for both, which contained 0.5 milligram of phenylpantothenone per cubic centimeter. Differential plate counts were made after an incubation period of 48 hours. Counts made at pH 7.0 showed the total numbers of both species; those done at pH 4.0 indicated numbers of *Saccharomyces*. When 0.04 gamma of pantothenic acid per cubic centimeter was present in the basal medium along with the phenylpantothenone, very little multiplication of either species was observed. *Saccharomyces* did increase slightly and to approximately the same extent that it did

when tested by itself. The amount of growth, however, was less than 1 per cent of that which occurred in the same medium without the analog. When the pantothenic acid content of the test medium was raised to 20 gammas per cubic centimeter and both organisms were inoculated into it, *Saccharomyces* was still inhibited to the same extent as was just indicated, but *Lactobacillus* grew normally (3×10^8 living cells per cc.). The selectivity depended on a difference in ability to realize antagonism in the two species.

In similar fashion, 4-aminopteroylglutamic acid in the presence of an excess of the related metabolite will not suppress growth of organisms for which the vitamin will reverse readily the action of the analog but will retard forms in which the antagonism is difficult to demonstrate.

A further illustration of a similar means of achieving selectivity is the finding that the multiplication of bacteriophage T2 within the cells of *Escherichia coli* can be inhibited by high concentrations of sulfanilamide (456). The toxic effects of the analog on the bacteria were almost nullified by additions of methionine, xanthine, valine, and thymine. These are non-competitive and structurally dissimilar antagonists of sulfanilamide and were sufficient to allow growth of the host cells. However, they were not sufficient to permit multiplication of the virus within the cells. This case differs somewhat from the one involving phenylpantothenone, because the selectivity depended on the differential effect of a number of non-competitive antagonists of the antimetabolite. *p*-Aminobenzoic acid, the structurally similar metabolite, overcame the action of the analog on both species. The bacterial cells were pictured as possessing several uses for *p*-aminobenzoic acid, the more vital of which were concerned with the formation of methionine, xanthine, valine, and thymine. Another function which was essential to multiplication of the virus was apparently not so to the growth of the bacteria, and this allowed the demonstration of a selective effect.

Possibility of selective action based on differences in combining power of the enzyme for the antimetabolite

According to the favored hypothesis to explain the mode of action of antimetabolites, the combination of the inhibitory agent with an enzyme, or other specific protein with which the metabolite normally reacts, is essential. If this enzyme is quite able to form such a complex with a given antimetabolite, while in another species such a union is poorly formed if at all, then the first organisms might be susceptible, and the second resistant, to the analog. For example, consider the

system of enzymes which forms cozymase from nicotinamide. If in some living things the specific protein to which nicotinamide is substrate should be capable of combination with β -acetylpyridine, then this latter substance should antagonize the action of the vitamin in these species. If in some others the protein which performs the same function is different enough from that of the first class so that no combination is formed, then the analog would not harm this second class of species. Actually, β -acetylpyridine does show a selectivity of action in that it competes with nicotinamide (or nicotinic acid) in higher animals but does not inhibit the growth of many kinds of microorganisms. This behavior is not correlated with nutritional requirement for the vitamin, and, furthermore, the resistant species have been shown not to destroy the analog (94). It may be that the relevant proteins differ in the two classes of organisms with respect to ability to combine with β -acetylpyridine (450). This postulate has not been tested experimentally and cannot be so tried until it is possible to carry out *in vitro* the formation of cozymase, or one of its intermediates, from nicotinamide.

Is there then any experimental backing for the general idea just outlined? If the relative combining powers of hemoglobins of various species for carbon monoxide and for oxygen are measured, the differences among them are astonishing. Although human hemoglobin has an affinity roughly 200 times greater for carbon monoxide than for oxygen, as one goes down the evolutionary ladder he finds that this value drops until with the protein of the invertebrate parasite *Gastrophilus intestinalis* the value is about 0.7 (252). Even among the mammals, the hemoglobins differ in this respect (232). It is clear, therefore, that the specific protein which is concerned with the transport of oxygen in various species does not have the same relative affinity for the antimetabolite carbon monoxide.

Selectivity based on ability of one organism to destroy the antimetabolite

If some organisms can destroy or excrete a harmful agent while others cannot, a sufficient basis for selective action would exist. We should still lack an understanding of why this should be so but would need to look no further for the immediate cause. Perhaps a large number of cases are in this category.

An interesting example of this is found with microorganisms which are resistant to pyriithiamine. Several of these apparently owe this character to the possession of a metabolic means of cleaving the harmful agent to yield the pyridine and pyrimidine halves from which it

is made. This capacity to destroy the antimetabolite is not shared by susceptible species (120). The cleavage of the analog is at the same position in the molecule as that at which thiamine itself is attacked by a naturally occurring enzyme. This seems of interest in view of the fact that the resistant forms are the ones which make their own thiamine, whereas the susceptible ones do not.

The usual means of detoxification of all sorts of harmful substances probably explain many instances of selective action of antimetabolites as well. Methylation, oxidation, and conjugation perhaps are at work with these as with other poisons to remove them from the sphere of action. In general, the possession of ability to destroy the harmful substance will explain only why isolated organisms differ in susceptibility but will not clarify selective action when the species are together. This principle was discussed earlier.

Selectivity based on differing functions of the metabolite in various species

In this section a case will be considered about which there is much uncertainty, or at least lack of unanimity, in the interpretation. The chief difficulty seems to arise from a lack of knowledge of the exact chemical structures of the participants, so that some investigators deny that the case belongs among the antimetabolites at all. Nevertheless, the situation was discovered as a result of application of principles learned from the antimetabolite phenomenon and seems to have superficial justification for admission. If we grant for a moment that the substance is an antimetabolite, we shall be able to see the possible reason why a selective action was found.

When the influenza virus is mixed with erythrocytes of species susceptible to the disease which it causes, the cells are agglutinated, and as they settle they take down with them the virus. If the mixture is incubated for a time, the virus reappears in solution, and the cells resuspend. Although the virus is not affected appreciably by the adsorption and elution, the erythrocytes are permanently altered. Some substance in them which is necessary to the adsorption of virus has been destroyed. These facts have been interpreted (316) to mean that the virus contains a special enzyme which attacks a specific substrate in the cell and destroys it. Adsorption of the virus thus represents the formation of an enzyme-substrate complex. The nature of the substrate is unknown, but evidence points to the conclusion that it contains a polysaccharide. Polysaccharides of tissues which the influenza virus normally attacks are usually composed in part of glucuronic acid residues. If this view of the facts is correct, then the enzymic attack

of the specific polysaccharide substrate of the cell by the virus should be capable of inhibition by a suitable analog of the substrate. Several polysaccharide materials, most of them rich in galacturonic acid residues, were found to cause this inhibition (258). Of these, apple pectin was the best. Not only would it retard the agglutination of erythrocytes by the virus, but it would likewise protect living animals (chicken embryos) from otherwise infective doses of the virus. The relative potency of a variety of carbohydrate-containing materials in causing inhibition of virus hemagglutination is shown by the data of Table 4,

Table 4

Effect of various carbohydrate-containing materials on hemagglutination by influenza A virus

Substance	Inhibitory Activity
<i>Polysaccharides</i>	
Apple pectin	+
Citrus pectin	+
Flaxseed mucilage	+
Gum acacia	+
Specific polysaccharide of acacia	0
Gum myrrh	+
Alginic acid	Trace
Soluble starch	0
"Starch polyaldehyde"	0
"Starch polyacid"	0
Agar	0
<i>Simple carbohydrates</i>	
Galacturonic acid	0
Cellobiuronic acid	0
Inositol galactoside tartrate	0
Galactose	0
Aldobionic acid of flax	0
Glucose	0
Mannose	0
Ribose	0

and the effects of two of these substances on multiplication of the virus in chicken embryos can be seen in Table 5. Both of these have been taken from published papers (258).

If apple pectin is acting as an antimetabolite to a specific polysaccharide in the cell, and thus protecting it from the influenza virus, then the metabolite should antagonize the effect of a fixed amount of the pectin, just as thiamine antagonizes a fixed amount of pyriethamine. Extracts of cells, if they contain this virus substrate, should thus over-

Table 5

Effect of apple pectin and of alginic acid on multiplication of influenza A virus in embryonated eggs

Substance	Amount, mg. per egg	When Given	Eggs	Eggs Showing Virus Multiplication
None			24	24
Apple pectin	50	Before virus	61	5
		After virus	56	17
Alginic acid	25	Before virus	4	1
	50	Before virus	16	14
		After virus	28	24

come the inhibition of virus hemagglutination caused by pectin and allow the attack on the cells which is signaled by complete agglutination. Properly prepared extracts of susceptible erythrocytes did have

Table 6

Antagonistic effect of an hemolysate of chicken erythrocytes on the inhibitory action of apple pectin towards influenza virus hemagglutination

Hemolysate		Apple Pectin, cc.	Phosphate Buffer, cc.	RBC Sus- pension, cc.	Virus Sus- pension, cc.	Hemag- glutina- tion *
Dilution	Cc.					
0	0	0	0.50	0.25	0	0
0	0	0	0.25	0.25	0.25	c
0	0	0.10	0.15	0.25	0.25	t
0	0.15	0.10	0	0.25	0.25	t
1:10	0.15	0.10	0	0.25	0.25	c
1:1,000	0.15	0.10	0	0.25	0.25	c
1:10,000	0.15	0.10	0	0.25	0.25	p
1:100,000	0.15	0.10	0	0.25	0.25	p
1:10	0.15	0	0.35	0.25	0	0

* c = complete, t = trace, p = partial.

this power to antagonize the effect of pectin, as the data of Table 6, taken from the literature (317), will show. The active material was purified somewhat and was found to be a non-dialyzable substance with

the qualitative behavior of a polysaccharide. Purified virus destroyed this partially purified cell substance when the two were mixed *in vitro*, so that it appeared to be a cellular component with which the virus reacted.

When the apple pectin was injected into chicken embryos, it proved to be quite harmless, and yet it hindered markedly the multiplication of the virus. To what was this selectivity due? In the animal, the virus receptor, or virus substrate, is at or near the surface of the cell. At least, pictures obtained with the electron microscope seem to favor this view. In the surface it probably plays the role of a morphological unit. Through this structure the virus probably must pass in order to gain entry into the cell. Outside, the virus has not been found able to multiply. It must, therefore, be vitally interested in attacking this structural unit with its special enzyme. If prevented from doing so by apple pectin, the virus cannot grow. On the other hand, the animal cell can endure with impunity an analog of one of its morphological units. So far as the animal is concerned, the metabolite in this case does not function by constant participation as substrate in a metabolic reaction. The selectivity of action of apple pectin would thus reside in the difference in function of the metabolite in the animal cell and in the virus.

As mentioned earlier, some investigators prefer to attribute the antiviral properties of a polysaccharide such as apple pectin to causes other than its being an antimetabolite (318, 319). To the present author, the evidence in favor of placing it among the antimetabolites seems better than any other explanation, but the evidence would be stronger if the exact chemical structures of the pectin and of the virus receptor were known. Since data are scarce, opinions have of necessity figured largely in the interpretation both of this aspect of the case as well as in that dealing with selectivity.

The influence of physical factors such as dissociation constants and distribution between aqueous and non-aqueous phases

In the transportation of an antimetabolite from the place of administration to the site of action, the physical properties of the compound may be of considerable importance. In so far as the route and the method of transport vary in different types of living things, these factors may influence the relative effects of a compound in two species or tissues. Usually, however, potency is influenced in a quantitative fashion by such factors, while qualitative properties remain the same. This can be seen from a consideration of the relationship to bacteriostatic potency of the acid dissociation constants of sulfanilamide deriva-

tives (320). Noting that bacteriostatic potency rose to a maximum and then declined in a series of sulfanilamide derivatives as the acid dissociation constant increased, Bell and Roblin concluded that only the ionized form of the drug was contributing to activity. This may be due to the failure of the unionized form to penetrate the cell, although this is certainly not proved. The *pH* of the environment in which a given microorganism was growing, therefore, influenced the relative potency of a series of sulfonamide drugs. If the acidity approached that at which a particular member of the series was undissociated, then this one might be less active than a second member of the series, while at another *pH* the reverse might be true. Situations of this sort were demonstrated experimentally (321). Thus, although sulfathiazole was much more active than sulfanilamide at *pH* 7, the two analogs were approximately equal in potency in the *pH* range between 9 and 10. Sulfathiazole is the stronger acid. Both derivatives, however, had typical sulfonamide activity, and the relative potency was the thing affected. Considerations of this sort seem to be important in realizing the most active members of a series of related compounds, but have not been a deciding issue in determining selectivity.

In ensuring the safe arrival of an antimetabolite at the site of action, its relative solubility in aqueous and oily phases may be important. Perhaps the selective action of some drugs on certain tissues of an individual in preference to other neighboring structures may be decided in this way. For example, this possibility has been presented by Albert (449) to help in the understanding of the selective toxicity of 8-hydroxyquinoline. The evidence consists of the observation that introduction of groups which make the quinoline more water soluble reduces the biological action very considerably. Similarly, the differential activity of substances behaving as plant-growth hormones in two different test systems has been correlated with distribution of the individual compounds between oil and water (323). In general, however, poor success has attended most attempts to demonstrate this idea experimentally. Limitations of the methods available for study of the problem probably contribute to this. It is, however, an aspect most investigators consider when they think of selective action.

Fixation to tissues

This is an aspect of the problem about which very little is known. What is certain is that some antimetabolites have the property of becoming fixed to organisms so that their effects are delayed, or are long continued. For example, a single oral dose of pyriithiamine given to a mouse causes no detectable harm for about a week. Then the signs

of thiamine deficiency appear in rapid succession, and, unless thiamine in excess is given, the animal dies. Approximately the same total quantity of the analog is needed to produce an effect whether it is given in one dose or distributed over many days. One can conceive that it has been able to do this by fixation to the tissues and exertion of its effect continuously.

Real evidence exists for the fixation of one analog of pantothenic acid to isolated sections of rabbit intestine (439). This substance is the *p*-nitroanilide of pantoyltaurine. When it was applied in solution to the tissue, it was able to prevent the synthesis of acetylcholine from choline (presumably by inhibiting coenzyme A formation from pantothenic acid). Repeated washing failed to dislodge it, and it remained fixed and capable of exerting its effect. It was one of the most powerful analogs tested. Possibly the ability to remain fixed contributed to this property.

If 3-3'-methylenebis-(4-hydroxycoumarin) is taken to be an antimetabolite, then it too is an example of an agent which is retained by tissues. A portion of an injected dose of the radioactive compound was still found in the livers of either mice or rabbits for several days after administration (468). The effect on the prothrombin level of the blood persisted as long as the compound could be detected in the liver and for about 3 days thereafter. Administration of large amounts of vitamin K resulted in a rapid clearing of the analog from the animals.

With two drugs not generally believed to be antimetabolites, the actual fixation and persistence of the compounds in the tissues has also been proved. A description of these may help in the understanding of similar situations with antimetabolites. Thus, with radioactive penicillin, radioactive material is firmly attached to the bacterial cell and is passed on to daughter cells when division occurs (326). Likewise, methylene blue, which competes with acetylcholine in causing contraction of an isolated frog heart, can be seen, by means of its blue color, to be fixed to the tissue from which prolonged washing does not dislodge it (279). Although the hormone and much of the methylene blue which antagonizes its action can be readily washed out, a part of the latter remains bound firmly. From this we must conclude that the binding is a process apart from the antagonism to the metabolite, although it may profoundly influence that antagonism.

Some empirical information is being accumulated to indicate the types of structural alteration of a metabolite which may be expected to give rise to long-acting analogs, and this will be discussed briefly in Chapter 10. The application of this knowledge to the problem of selectivity of action can only be conjectured but may lie along the path

indicated in the above section on selectivity based on variation among organisms of ability to demonstrate antagonism with the metabolite.

Fixation to tissue does not seem to be synonymous with irreversible antagonism. The cases of pyrithiamine and of methylene blue illustrate this, because these compounds apparently are bound to tissue and yet are competitive antagonists of their related metabolites. Nevertheless, some of the types of structural alterations which give non-competitive or irreversible analogs likewise are the ones associated with the formation of these long-acting substances.

High potency is frequently associated with those antimetabolites which seem to possess this property of fixation to tissues, especially if they also show antagonism of the non-competitive type. This fact can be seen from consideration of the relative potency of analogs of folic acid. For this comparison either a bacterial species or a higher animal will serve as the test system, but let us look only at the results with rats. The data in Table 7 show that for those analogs the effects of which

Table 7

Potency for rats of antimetabolites of folic acid correlated with reversibility by the metabolite

Analog	Structural Alteration	Antagonism by Pteroylglutamic Acid	Minimal Toxic Dose,* gammas per day
Pteroylaspartic acid	Loss of a $-\text{CH}_2-$	Competitive	Greater than 1,500
7-Methyl pteroylglutamic acid	CH_3 for H	Competitive	10,000
9-Methyl pteroylglutamic acid	CH_3 for H	Competitive	10,000
7-Hydroxy-9-oxypteroylglutamic acid	OH for H, CO for CH_2	Competitive	2,000
4-Aminopteroylglutamic acid	NH_2 for OH	Absent	10
4-Aminopteroylaspartic acid	NH_2 for OH, loss of $-\text{CH}_2-$	Present	3,000

* Determination on weanling animals fed a folic acid-deficient ration.

folic acid readily counteracts, the amount needed is of an entirely different order of magnitude than for those which are not readily reversible by the vitamin. Those which are not readily reversible by the

vitamin are far more active, and they are the ones which are believed to be fixed to the tissues which they affect.

A somewhat similar situation can be seen with other kinds of antimetabolites. Reference to Table 6 of Chapter 1 shows that the highly active analogs of pantothenic acid are those with which some difficulty is encountered in causing reversal with the vitamin, i.e., pantoic hydrazide and phenylpantothenone and its close relatives. Again, consider the analogs of phenylalanine which were described in Table 3 of Chapter 5. Chloromycetin is far more active than β -hydroxyphenylalanine or 2-aminophenylpropyl alcohol.

Why this should be so is easy to understand. The substances which are fixed to the site of action and which are antagonized with difficulty by the related metabolite do not have to be present in overwhelming concentration in order to displace or exclude the normal metabolite from its specific sites.

If tissues or species vary widely in their ability to fix an antimetabolite but are in other aspects of selectivity the same, then the ones which bind the compound are liable to be the ones affected. This is a possibility which has not yet knowingly been exploited experimentally. However, the anchoring of drugs to pathogenic microorganisms was considered by Ehrlich to be of great importance to chemotherapy, and subsequent experience with compounds containing arsenic and antimony, even though they may not be antimetabolites, has justified belief in the idea.

CHAPTER 7

Applications to chemotherapy

Since its inception the whole field of antimetabolites has been much enmeshed with attempts to apply its agents to problems of practical chemotherapy, and for this reason it has not been possible entirely to postpone references to these in the preceding chapters. Thus, for example, the application to chemotherapy is inherent in any study of the basis of selective action of a compound on two species growing together. However, in this chapter, attention will be centered on the question of what relationship antimetabolites may have to chemotherapy aside from possible chance discoveries that they are useful there. What we may call the premeditated successes in the production of useful agents have been few indeed. Most of the accomplishments are experimental models which may in future show how such drugs are to be formed. Thus far, they have dealt mainly with diseases experimentally induced in laboratory animals. Time will be required for the dissemination of the information so collected and consequently for the adequate trial of its applicability to the problems of practical chemotherapy.

For the present discussion, the field of chemotherapy can be divided into two sections: namely, that dealing with infectious diseases and that treating with non-infectious disorders. In the former, the aim is to destroy, or at least to inhibit the multiplication of, a foreign organism which is growing in the tissues of an animal. In the second, an attempt is made to control the activities of integral parts of the individual which is under treatment. It is not clear whether this division is superficial or fundamental, but at present it serves to orient the direction of most of the efforts.

The idea that antimetabolites might be useful therapeutic agents in infectious diseases arose from the demonstrations that *p*-aminobenzoic acid was concerned in the functioning of one group of existing drugs (34, 283). Because sulfanilamide and its congeners were practical and useful medicinal substances, the action of which was antagonized by *p*-aminobenzoic acid, other antimetabolites were expected similarly to be useful. Experience has shown, however, that this does not necessarily follow.

Nevertheless, the idea of antagonism between structurally similar compounds, and of its potential usefulness in chemotherapy, is abroad, and stalks through all sorts of efforts in this field. However, the knowledge of how best to apply the principles of the antimetabolite phenomenon is rudimentary. Therefore, quite varied working hypotheses have actuated the attempts, and it is uncertain which, if any, of these are right. Despite this uncertainty, these attempts are being made. They rest securely on the undisputed fact that a pair of structural analogs may show opposing biological effects.

***p*-Aminobenzoic acid in the treatment of experimental rickettsial infections**

One of the first uses of the phenomenon of competition between structurally similar compounds in order to arrive at a therapeutic agent was the demonstration that *p*-aminobenzoic acid would cure mice of experimental infections with murine typhus. Having observed in routine testing of drugs that sulfanilamide and its derivatives intensified rather than alleviated experimental typhus infections in these animals, Snyder et al. (327) reasoned that *p*-aminobenzoic acid might therefore be expected to have the opposite effect and thus to control the disease. Trials then showed that it did. Large amounts of *p*-aminobenzoic acid, when injected or fed to animals previously inoculated with ten lethal doses of the rickettsiae made possible the survival of nearly all the individuals. The large dose of the compound which was required was a serious handicap to practical application of this finding, and, although search was made for more active derivatives, none was found. *p*-Aminobenzoic acid itself was therefore tested in clinical typhus fever, and beneficial results were reported (328). The large quantity of the drug required would seem to mitigate against its widespread practical use.

In experimental animals, *p*-aminobenzoic acid proved capable of exerting a therapeutic effect not only on murine typhus but on certain other rickettsial diseases as well. For example, the multiplication of the organisms of typhus in chicken embryos (341) or of scrub typhus

in such embryos or in rodents was inhibited by large doses of the compound (329).

The explanation of the chemotherapeutic effect of *p*-aminobenzoic acid is not clear. Although this metabolite, when it acts as a growth factor for bacteria, is effective in extremely small amounts, very large doses are needed for the antirickettsial manifestations. In high concentration, the substance is toxic to many kinds of bacteria and fungi as well as to rickettsiae. Why it should be so and why it should be less poisonous to higher animals than to these microbes are entirely unknown. Only the circumstances surrounding the discovery of its chemotherapeutic effect were connected with antimetabolites.

Experimental treatment of induced microbial infections with antimetabolites

After the discovery that sulfanilamide and its derivatives inhibited bacterial growth in competition to *p*-aminobenzoic acid, antagonistic analogs of several other growth factors were tested for ability to control induced bacterial infections of laboratory animals. Small success attended these efforts, due in part to failure to appreciate fully the need for selective action or the means by which it was to be achieved. Other reasons of which we still know little probably contributed as well. Nevertheless, these failures demonstrated adequately that useful agents could not be produced merely by making analogs of vitamins. Likewise, refinements of this idea, such as the determination of nutritional requirements of the pathogen, and concentration of efforts on the formation of analogs of those growth factors which it contained or required in the medium, did not meet with success. Some investigators proceeded on the premise that selectivity of action was not important, because, even though an analog might induce a deficiency disease in the host as well as in the parasite, the latter might succumb first. The shorter life span of the parasite was quoted in support of this idea. Once freed of the invader, the harm done to the host could then be corrected by administration of the competing vitamin. This idea has not been substantiated by the production of an agent which will quench an infection and allow recovery of the host. It would seem that more insight is required into the processes of growth and parasitism before this approach to the treatment of bacterial infections will yield useful drugs (248, 330).

One incentive which kept interest alive was the ability of pantoyletaurine (thiopanic acid) to save rats from fatal infection with hemolytic streptococci (174). Although thus effective in rats, in mice this analog of pantothenic acid exerted no beneficial action against the same bac-

teria. The level of pantothenic acid in the blood of mice was then shown to be greater than in rats, and hence the conclusion was drawn that a low blood level of this metabolite was required before any chemotherapeutic demonstration could be made. The greater amount of the antagonistic vitamin in mice apparently overcame any toxicity of the agent for the pathogens. Still, however, even for rats the dosage required was excessive, both in amount and in frequency of administration. Furthermore, it was not established whether the analog would completely eradicate the infection, or merely suppress it for a limited period of time. Efforts were then made to find more powerful members of the series of which pantoyltaurine was the first, and to this end large numbers of pantothenic acid analogs were tested. The dibromoanilide of pantoyltaurine did prove to be much superior in that with moderate doses it was capable of control of streptococcal infections induced in mice (185).

Even more promise of success was offered by the findings in the control of experimental malaria. A new type of analog of pantothenic acid, namely, phenylpantothenone, had been produced, and shown to affect adversely the growth of a wide variety of microorganisms. At the same time it proved relatively harmless to animals (169). In view of its high activity and its selective action, it was tried in experimental malarial infection of ducks, where it was found to be equal to quinine (186). Experiments in other species proved that in these as well phenylpantothenone would control the disease caused by transferral of infective blood. In man it also showed this power. Whether or not it will control relapsing malaria as seen clinically has not been determined, but it seems a worth-while project to investigate.

These analogs of pantothenic acid probably owe some of their therapeutic effectiveness in experimental infections to their inability to call forth pantothenic acid deficiency in animals. It was surprising that this should be so, and the reason for this selectivity is still unknown. The advantage of making use of it is undeniable.

Another aspect of the therapeutic effect of both phenylpantothenone and the dibromoanilide of pantoyltaurine is that some difficulty is experienced in reversing their effects with the related vitamin. With phenylpantothenone, antagonism can be shown only with those species which require pantothenic acid as a growth factor. Much empirical observation would suggest that difficulty of reversal of the action of an antimetabolite by the metabolite is a desirable, if not even an essential feature, in therapeutic agents. Most of the antimetabolites which have found practical application, e.g., chloromycetin, 3,3'-methylenebis-(4-hydroxycoumarin), dichloronaphthoquinone, hexachlorocyclohexane,

4-aminopteroylglutamic acid, and some of the antihistamine drugs¹ have this property, although the sulfonamide drugs do not.

Antitubercular activity of *p*-aminosalicylic acid

One drug which has found some application in the treatment of clinical disease of bacterial origin was discovered partly through knowledge of the antimetabolite phenomenon. This was *p*-aminosalicylic acid which has been used in conjunction with streptomycin in attempts to control clinical tuberculosis. This analog of *p*-aminobenzoic acid is quite harmful to the tubercle bacillus and is relatively non-poisonous to higher animals (144). Large doses have been used in tubercular patients and in infections of laboratory animals with the tubercle bacillus, and have given a desirable therapeutic effect, although not an entirely satisfactory one. However, it was sometime after its discovery before its antimetabolite relationship to *p*-aminobenzoic acid was recognized. Rather, the drug was found by testing structural analogs of benzoic acid and of salicylic acid. The reason for choice of these latter compounds was the observation that *in vitro* the tubercle bacillus oxidized them. Consequently, substances were sought which would have the opposite effect and would retard oxygen uptake by the bacteria. When *p*-aminosalicylic acid was observed to do this, and when it chanced to be well tolerated by animals, the therapeutic trial of it was initiated. At the present time, sufficient experience with it is not available to determine adequately its ultimate usefulness.

Influence of certain polysaccharides on experimental infections with pneumotropic viruses

The multiplication of influenza A virus in the allantoic sacs of embryonated eggs can be almost completely stopped by administration of apple pectin. Data to illustrate this fact have been given in Table 5 of Chapter 6. The reason for touching on these experiments again is that they indicate one way in which knowledge of the antimetabolites has been applied to problems of chemotherapy. At the time they were carried out, no substances (aside from specific antibodies which occasionally were successful) were known which would markedly retard the multiplication of virus diseases without profoundly injuring the host animal. Many attempts had been made to find such materials, and these had included routine testing of existing antimetabolites. Some justification for such random trials was offered by the belief in the

¹ Opinions have differed as to whether some of the substances mentioned are antimetabolites, but in previous chapters (1 and 5) the evidence bearing on the question was discussed and seemed sufficient to rate them at least as doubtful examples.

obligate nature of parasitism involving viruses. These intracellular pathogens were thought to share some of the vital metabolic processes of the host cell, and it was argued that an antimetabolite might be found which would interfere in such a reaction, and thus hinder the parasite. However, no reason has been given why this might not be as harmful to the host as to the virus. Reliance was put on chance that a selectively toxic compound might be found, but this was not realized. When, however, the knowledge about the mode of action of antimetabolites was applied to what was known of interaction between susceptible cells and the influenza virus, an active substance (apple pectin) was soon found in the manner described previously.

Although this polysaccharide was able to check the infection in embryonated eggs, it was not active in saving mice from death after installation of influenza virus. Oral, parenteral, or intranasal administration of the pectin were all unsuccessful. From the nature of the disease in mice, the intranasal route of therapy might offer most promise for a non-diffusible substance such as pectin. Trials by this route were unsatisfactory because of the small amounts which could be introduced. In the egg experiments about 25 milligrams of it were required for each individual but only a tenth of this quantity could be given intranasally to mice. Unless a more active derivative can be made, therefore, the demonstration may be confined to one species under rather artificial laboratory conditions. The practical applications of substances which show promise in one animal species have frequently proved difficult.

Polysaccharides which would protect other living things from destruction by other viruses were soon found. One such example was the protection of *Escherichia coli* with citrus pectin from lysis by bacteriophage (457). The basic idea which led to this finding was similar to that which pointed to apple pectin as an inhibitor of influenza virus. Evidence existed to suggest that the phage attacked the bacterium through a specific polysaccharide receptor substance, and it was postulated that an analogous molecule, such as pectin might be, would thus present a difficulty to the parasite. Actually, the phage multiplied in bacterial cultures protected by the presence of citrus pectin, but the host cells were not destroyed as they normally would have been. No clear evidence was found to show whether the polysaccharide acted as an antimetabolite in bringing about this protection. It may possibly have functioned quite differently.

At the same time that the effect of apple pectin in influenza virus was found (258), Horsfall and McCarty (331) observed that another pneumotropic pathogen, the pneumonitis virus of mice, was retarded

in its harmful effects on these animals when certain polysaccharides were installed intranasally. Apple pectin did not prove active with this infection, but polysaccharide-rich preparations from streptococci or from Friedlander's bacillus were effective. These findings were not made with any thought of antimetabolites but were an accidental discovery. Subsequent trials showed that the substance from Friedlander's bacillus inhibited the multiplication of mumps virus in embryonated eggs (319). Continued experimentation, unfettered by the working hypothesis surrounding the influenza model, led to conclusions quite similar to those attained there, namely, that the polysaccharides blocked some metabolic process essential for the continued growth of the virus (458). Whether or not structural analogy of the Friedlander's polysaccharide to an essential metabolite plays a role in this blocking has not yet been demonstrated.

Therapeutic effects of certain pyrimidine derivatives on experimental malaria and vaccinia

Recently, a few pyrimidine derivatives have been found which exert a powerful therapeutic effect on experimental malarias, and another of these compounds has shown some protection against infection of mice with vaccinia virus (the virus of smallpox vaccine) (491-494). These substances were developed as a result of attempts to alter the structures of the known metabolites which are pyrimidines in such a way as to achieve useful agents against infectious diseases. Because uracil, cytosine, and thymine are built into nucleic acids, and because these latter, especially the desoxynucleic acids, are thought to be of importance in cell division, antimetabolites of these pyrimidines were believed to offer some promise as therapeutic agents in infections. A large number (several hundred) accordingly, were screened for a variety of such effects. As a beginning, the ability of an analog to inhibit the growth of *Lactobacillus casei* cultured in chemically defined media was determined. Thymine is an essential growth factor for this organism under specialized conditions, and pteroylglutamic acid can replace this pyrimidine, presumably because of participation in the biosynthesis of thymine.

A group of substances, each one a 2,4-diamino-5-aryl-6-alkypyrimidine, was found to be toxic to *L. casei* when the growth of this organism was being supported with pteroylglutamic acid. When thymine was present the toxicity was greatly reduced (491). These analogs showed high potency when tested against blood-induced malaria of mice (*Plasmodium berghei*) and of other laboratory animals. The most active was the 5-(*p*-chlorophenyl)-6-ethyl analog, the structure of which is

shown in Figure 1. As little as 1 gamma per day sufficed to protect mice against the infection as evidenced by absence of parasitemia. A similar series of 2,4-diamino-5-phenoxy-6-alkylpyrimidines exhibited similar, but less marked, action (492). Fortunately, the toxicity of these kinds of analogs for higher animals was sufficiently low so that the therapeutic effects could be demonstrated without equivocation.

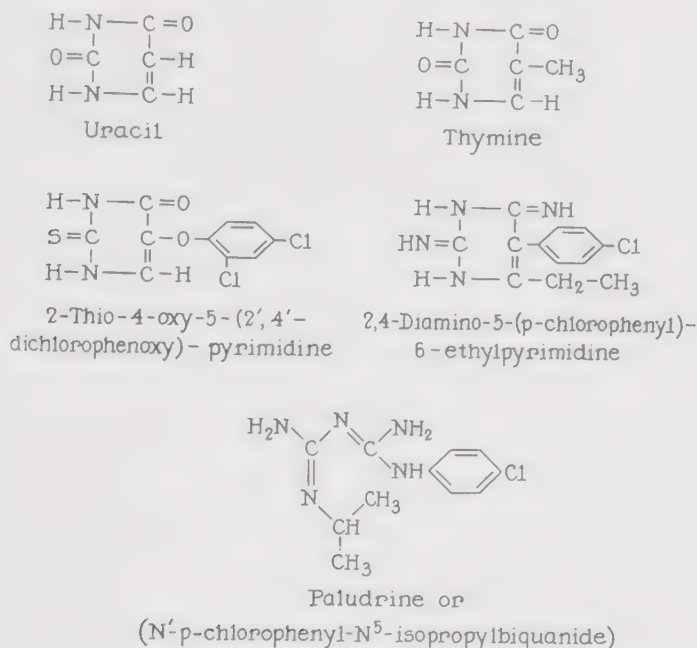


Figure 1. Some metabolically important pyrimidines and some anti-infectious agents similar to them.

If the therapeutic effect of these compounds is due to an antimetabolite action, it is not clear which metabolite they antagonize. Although the growth inhibition observed with *L. casei* *in vitro* was overcome by pteroylglutamic acid, the structural resemblance of this metabolite to the toxic agent is remote indeed. In the same *in vitro* bacterial test, the presence of thymine greatly reduced the effect of the substances. The compounds do resemble this metabolite, and the alterations involved in passing from it to them are similar to those which have proved efficacious in converting other metabolites into their anti-metabolites. The antagonism between thymine and these alkylphenyl-diaminopyrimidines, however, is apparently not competitive. This does not, of course, mean that they are not antimetabolites of thymine but does raise some question.

Another kind of antimalarial drug was conceived largely as the result of attempts to form analogs of metabolically important pyrimidines.

These are the biguanides (496). The structure of one of the most active, paludrine, is shown in Figure 1. Their discovery preceded the work just described, but their effects on the growth of *L. casei* have been shown to be antagonized by pteroylglutamic acid (495). This fact was the reason for testing the alkylphenyldiaminopyrimidines as anti-malarial agents.

The testing of a large number of pyrimidine derivatives for ability to restrict the growth of vaccinia virus led to the finding of one which was able to protect most mice against about 100 fatal doses of this infectious agent installed intracerebrally (494). This compound was 2-thio-4-oxy-5-(2',4'-dichlorophenoxy)-pyrimidine. Its resemblance to uracil and to thymine is apparent (see Figure 1), but its metabolic involvement with these metabolites has not yet been established. Just as in the antimalarial pyrimidines, this analog was less toxic to the higher animals than it was to the infectious agent. The reasons for this are still obscure. Nevertheless, substances which will show a therapeutic action against viral infections are so rare that this one is outstanding, although it is still a laboratory model.

Experimental models in the chemotherapy of non-infectious diseases

Although the name chemotherapy has come to acquire a connotation implying treatment of infectious diseases, this branch of the subject is only a part of it. The brilliant successes of agents such as the sulfonamide drugs and penicillin and the intensive search for antibiotic substances have tended to obscure the aspect of chemotherapy which deals with maladies of non-infectious origin or propagation. Aside from drugs which were discovered by chance and subsequently shown to be antimetabolites, no substances of practical usefulness in such diseases have been produced. Rather, a group of experimental models has been developed through the use of antimetabolites, and these serve only to show some of the things which can be done. They may not *per se* have practical usefulness.

Prediction of new series of pharmacological agents

After the discovery of the effects of the antivitamins on animals, it became clear that a means was at hand for the prediction of new types of pharmacologically active substances (205). Usually the first member of a series of drugs is found by chance. Such was true with atropine and with many other medicinal substances known today. After this first member is established, better ones may be made by alteration of its structure in order to increase potency or decrease toxicity. In the same

way, certain other desirable features of its action may be accentuated and undesirable ones repressed. So with atropine; better drugs have been made by changes which have given such agents as trasentine and related spasmolytic compounds (332). However, the application of a knowledge of antimetabolites may make it possible to eliminate chance as a sole key to the first member of a series and enable predictions of its nature to be made. To do this one must draw upon the accumulated knowledge of the changes characteristic of deficiency of various metabolites. Knowing that a given pharmacological sign is a feature of the lack of, let us say, one vitamin, we may anticipate that a properly constructed analog of that vitamin will call forth this manifestation. From the discussions of Chapter 10 it is plain that the types of structural alteration which might be tried are beginning to be understood, and that the choice of the proper analog is not entirely left to chance. With the correct antimetabolite in hand, we should then have the first member of a new series of pharmacological agents. With this first member of the series as a starting point, desirable modifications to potentiate its activities could be made in the classical fashion.

We should not be surprised if some of the signs of a deficiency disease are desirable or useful. A backward glance at a chance discovery may clarify this (205). 3,3'-Methylenebis-(4-hydroxycoumarin) will call forth in animals an increase in the clotting time of the blood, which is a characteristic sign of vitamin K deficiency. Whatever usefulness this drug has is due largely to its ability to elicit this sign of deficiency. If now, there were no agents to prolong the clotting time, and if this prolongation were known to be a sign of vitamin K deficiency, the formation of an antivitamin K would seem to be the logical way to get one. In the examples to follow, the working out of this idea in experimental models, and to a limited degree in clinical trial, can be seen.

α -Tocopherol quinone

In rats or mice a deficiency of vitamin E manifests itself not by failure of growth, or by death of the animals, but rather by an effect on pregnant females. This is the major manifestation of the deficiency, although in rats, but not in mice, testicular damage in males may also be found. In females, pregnancy is established in the absence of the vitamin, and it progresses for the first two-thirds of the gestation period. The embryos then die and are resorbed. This resorptive interruption of pregnancy is the characteristic sign in these species. Other manifestations, such as the development of muscular dystrophy, usually require a period of deprivation lasting for at least a generation, although

in other rodents they may be more prominent. These characteristics of the lack of this vitamin made tocopherol seem an apt choice to test the ideas previously outlined.

α -Tocopherol quinone proved to be capable of calling forth in mice signs similar to those seen in vitamin E deficiency (127). Given to pregnant mice at about the fifth day of gestation, large doses of this substance produced death of the embryos at the fourteenth to sixteenth day, followed by their resorption. At this time also, severe bleeding from the reproductive tract occurred in some of the animals. This hemorrhage was atypical of tocopherol deficiency of dietary origin because there it could only be seen histologically. The effects of the analog were not due to permanent damage because, after such an episode, mice were able to proceed normally through subsequent pregnancies. Non-pregnant individuals were not harmed detectably by the analog. Although α -tocopherol quinone is closely related in structure both to vitamin E and to vitamin K (see Chapter 1), only the second of these metabolites was able to overcome its effects. Other analogs of vitamin K, which were not close relatives of vitamin E, did not show the same properties as did α -tocopherol quinone when tested in mice. The large dose (200 milligrams per mouse) of this substance which was necessary to produce an effect would indicate that much more active members of the series than this first one will be needed to make a good demonstration. Nevertheless, α -tocopherol quinone will cause resorptive interruption of pregnancy in mice and will selectively affect only pregnant individuals.

Analogues of thyroxine

Some diseases are believed to be due to the existence of an excess in the body of one of the hormones. This excess may result from either the increased production, or diminished rate of destruction, of the hormone in question. Thyroxine is such a substance. Some investigators have concluded that a naturally occurring disease, namely thyrotoxicosis, results from too much of this hormone. If this be true, an antagonist to this metabolite might be able to combat it. This is an elementary statement of the case, which may be attacked from many quarters but which has some support.

The analogs of thyroxine which were described in Chapter 1 were prepared partly in order to test experimentally whether an excess of the hormone could be so nullified. They were shown to antagonize the action of toxic amounts of thyroxine administered exogenously to tadpoles. This effect was seen most clearly in protection against the lethal

action of administered thyroxine, but it could also be noted in a retardation of the accelerated metamorphosis of these animals (128). Data illustrative of this are shown in Table 1 and in Figure 2. Subse-

Table 1

Effects of the *p*-nitrobenzyl ether of N-acetyldiiodotyrosine on the toxicity of thyroxine for tadpoles. (Measurements were made 2 weeks after treatment.)

Analog, milligrams per cc.	Thyroxine, gammas per cc.	Number of Animals	Number of Deaths	Remarks on Metamorphosis
0	0	74	3	No change
0	0.5	31	12	All had 4 legs
0	1.0	24	16	All had 4 legs
0	1.2	19	16	All had 4 legs
0	2.0	31	29	All had 4 legs
0	3.0	22	22	All had 4 legs
0.50	0	19	0	5 had hind leg buds or legs
0.75	2.0	12	1	Hind legs only
0.50	2.0	24	7	All 4 legs
0.37	1.0	23	8	All 4 legs
0.25	2.0	7	6	All 4 legs
0.25	1.0	11	6	All 4 legs
0.50	4.0	7	4	All 4 legs
0.50	0.5	12	0	Hind leg buds only
0.50	10.0	12	11	All 4 legs, but survival 1 day longer than controls which received 10 gammas per cc. thyroxine alone

quently some of these analogs were found to lower the metabolic rate of higher animals. Whether they can combat endogenously formed excesses of the hormone, such as are believed to cause the clinical disease, remains to be seen.

Analog antagonistic to folic acid

One of the striking features of a nutritional deficiency of folic acid is the marked leucopenia which accompanies it. White blood cells may fall to a very low level. Since the leucemias are diseases frequently characterized by exactly the opposite manifestation, the formation and testing of analogs of folic acid as agents to combat these diseases seemed a logical application of the idea previously outlined in this section (333). Those antifolic acids which have been so tried were selected on

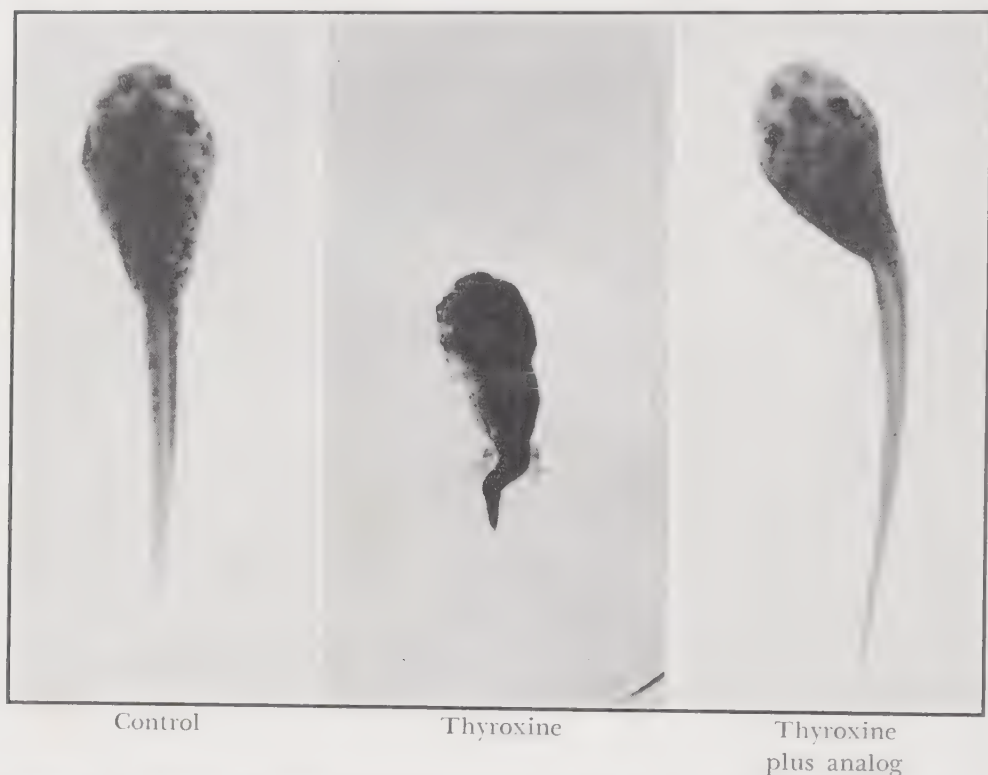


Figure 2. Tadpoles 10 days after exposure to thyroxine and to thyroxine plus the *p*-nitrobenzyl ether of N-acetyldiiodotyrosine. (Reproduced with the kind permission of the editors of *Growth*.)

the basis of their toxic, i.e., growth-inhibiting, powers for lactic acid bacteria. This criterion was chosen as the simplest way of finding those substances which antagonized the action of the vitamin. Highly active ones were tested in the leucemias. Whether this is an adequate way in which to choose the most favorable substance is open to debate. Nevertheless, the highly active 4-aminopteroylglutamic acid, or aminopterin, was first tried both in transplanted leucemias of experimental animals and in the disease as it occurs in children (226, 285). In animals, properly adjusted doses showed the ability to protect against the early lethal effects of the transplanted disease. Claims were made that remissions of the human disease were produced by it. The analog was, however, very toxic, and in higher animals its harmful effects were not remedied by folic acid. The divergence between the effective dose and the lethal one was not great enough to make it a safe drug. Furthermore, the clinical experience with it in children indicated that the curative effects did not last and that, after a year or two, no further response could be elicited. Perhaps the malignant cells became resistant to its action just as they apparently do to urethane, or as tubercle bacilli do to

streptomycin. Efforts were made to reduce the toxicity and, if possible, to enhance the activity by making other members of the series, and to this end 4-aminopteroylglutamic acids with methyl groups in place of hydrogen atoms at various points in the molecule were examined (334), as well as most of the analogs of folic acid shown in Table 5 of Chapter I. None of them has yet proved to be satisfactory, although reduced toxicity does seem to be attainable. The analogs which do not have the hydroxyl group in position 4 replaced by an amino group, and which are quantitatively far less potent in causing signs of folic acid deficiency of bacteria or of animals, have appealed to some investigators because of the ease with which their effects can be antagonized with the vitamin. One of these, the 7-(or 9-)-methylpteroylglutamic acid, has been reported to control experimental leucemia of mice (335).

In this instance, as with the early attempts at chemotherapy of bacterial diseases, perhaps too little attention has been paid to the problem of achieving a selective agent. If a drug kills the animal by creating an acute deficiency of folic acid, even though it may kill malignant cells slightly faster or in slightly lower concentration, the chances of its being a useful agent are small. Possibly more progress would be made in this direction if an adequate way of detecting a selective agent for this purpose were in existence.

Attempts to inhibit the growth of transplanted lymphosarcoma with other antivitamins

One of the signs of pyridoxine deficiency appears to be a retardation of lymphoid function. This is seen in deficient animals as an atrophy of the thymus and as a failure to form antibodies in normal amounts in response to an antigen (336, 337). Desoxypyridoxine administered to rats called forth manifestations similar to these, as one might expect an antipyridoxine to do. This suppression of the activities of lymphoid tissues by the antimetabolite was seen to be possibly what was desired in the control of lymphosarcomata, and hence desoxypyridoxine was tested in mice and in rats bearing transplanted tumors of this kind (102, 103). It was found to retard the growth of such transplants, and in some individuals, to bring about disappearance of the palpable tumors. However, when the drug was no longer given, the cancers reappeared. In rats, mere dietary deficiency of pyridoxine brought about effects on the lymphosarcoma similar to those induced by desoxypyridoxine (338). No selective action of the antimetabolite was therefore discernible.

Two antimetabolites related to riboflavin have been shown to allow survival of some mice bearing a transplanted lymphosarcoma which was uniformly fatal to untreated controls (339). Since a dietary de-

iciency of riboflavin produced results essentially the same as those with these antimetabolites, the action of the substances, isoriboflavin and galactoflavin, seems to have been only the induction of the vitamin deficiency state, which they had previously been shown to do. The testing of riboflavin analogs was prompted in this case by the realization that the return of the tumors in mice treated with desoxypyridoxine after the drug was stopped may have been due to the failure of antibody formation. The permanent suppression of the tumor is believed by some to depend on an antibody response of the host to it. If this be true, then a drug such as desoxypyridoxine, which interferes with antibody formation, would not be expected to bring about permanent cure. If desoxypyridoxine prevented the formation of antibodies, the tumor would thus grow again even though it had been made to regress by a short period of administration of the antimetabolite. With the riboflavin analogs, however, those mice which were able to survive the first month of treatment were able to live on, at least for a month or two, when the agent was withheld. The antimetabolites of riboflavin were thus an improvement over the one to pyridoxine but were still too toxic *per se*, and too lacking in ability to cure all individuals treated, to allow anything but a laboratory demonstration of their effects. Perhaps these demonstrations will serve as starting points for the development of selective agents which will control this type of disease, without harming the host unduly. For the present they are just laboratory models.

With the success and the shortcomings of the preceding experiments as guides, a new analog of riboflavin was produced which did not act as an antagonist to this vitamin in bacteria (*Lactobacillus casei*) or in higher animals, but which was said to cause regression of a transplanted sarcoma in mice (497). The substance was 6,7-dichloro-9-sorbitylisoalloxazine which differs from the vitamin in having chlorine atoms in place of methyl groups and a sorbityl side chain in place of a ribityl group. Details of its therapeutic virtues have not yet been published.

Triazolopyrimidine analog of guanine

Appreciation of the need for selectivity figured largely in the next attempt to produce an agent which would control malignant disease. When the triazolopyrimidine analog of guanine, a compound which differs from this metabolite only in that carbon atom 8 has been replaced by nitrogen, was given to mice bearing certain kinds of spontaneous or transplanted tumors, these growths were held in check (340). No cure was effected, because the cancers did not regress or disappear. However, the analog seemed relatively harmless to the host animals, and thus showed a specificity which the above mentioned antimetabo-

lites related to folic acid did not possess. The reason for selection of the triazolopyrimidine analog of guanine was the speculation, unsupported by any experimental data, that tumor cells had a nutritional need for guanine, whereas normal cells did not. If this should prove to be true, then the selectivity of this analog in hindering the malignant, but not the normal cells, would be correlated with the difference in nutritional requirements of the two kinds of living matter. Whether or not this particular substance will be of any practical therapeutic value is open to question not only because of its failure to cause regression of the disease but also because only a few types of tumors in mice were affected by it. It was not a generally acting carcinostatic substance. Most probably it will be only a laboratory experimental model.

Both in the work with folic acid analogs as well as in that with the relatives of guanine, one sees clearly that a metabolite was selected because it seemed to have an intimate role to play in the disease process. Both folic acid and the purines are believed to function in the formation of nucleic acids, and this process is postulated to be intimately linked with the disease. Such attempts to choose a metabolic reaction which may be primarily concerned in the disease seem wise. The indiscriminate choice of metabolites from which to derive specific agents would seem to be a return to reliance on chance.

These are some of the seeds which the phenomenon of antimetabolites has sown in the dark field of chemotherapy. Time must elapse before anyone can say whether they will come to fruition.

CHAPTER 8

Other applications to pharmacology

Pharmacology is so intimately linked with chemotherapy that to have discussed the applications of antimetabolites to chemotherapy is almost to have considered their application to pharmacology. However, some corners of the latter field, such as those treating of theoretical aspects of the action of drugs, remain. The insight which studies with antimetabolites have given about these matters will be discussed in this chapter.

Explanation of the mode of action of some drugs

Although there probably is no single comprehensive explanation of the mode of action of various drugs, the effects of a few classes of pharmacological agents can be understood, at least in part, if they are viewed as antagonistic analogs of certain metabolites. Some groups of common drugs, of which the individual members show similar pharmacological properties and bear structural resemblances to each other, can be seen to be related to a recognized metabolite. What is more, this metabolite will antagonize the effects of the drugs. In addition, many of the features of the pharmacological effects of the drugs are those which might be anticipated from a lack of the metabolite. These facts are the basis for the opinion that such drugs are antimetabolites and function primarily in the manner already depicted for such substances. The uncertainty arises when the antagonism of drug and metabolite is of the non-competitive type, or when the structure of the drug is too far removed from that of the metabolite. Uncertainty may also arise if the drug causes some, but not all, of the manifestations to be expected from deficiency of the metabolite, or if the metabolite over-

comes some of the effects of the drug, but not all of them. The working of these factors in the case of a few common drugs will be discussed in the next sections. Only a few instances will be described in order to illustrate the idea. These have been chosen because, to the present author, they seem to be some of the least complicated situations. A variety of other classes of drugs has at one time or another been postulated to act as antagonists to metabolites. In fact, this way of regarding the mode of action of drugs is beginning to enjoy a popularity which is not always critically founded. Perhaps in many cases the right explanation may prove to be that they either do not act as antimetabolites or that they are concerned with metabolically essential substances still unknown at present. Such a small number of metabolites are known in contrast to the actually functioning number which may be very large. To attempt an understanding of all those types of drugs which may prove to be antimetabolites would thus appear currently impossible. However, the evidence seems strong that some classes do act as antagonists of well-recognized metabolites.

The entire pharmacological effects of a compound probably do not reside in one type of action. It is common to find, for example, that antihistamine agents may show spasmolytic and anesthetic properties. To understand completely the pharmacological action of one substance may therefore be unattainable with one postulated mechanism, even though a large part is thus clarified.

The antihistamine drugs

To the author of the present book (118) and to a few others (76) most of the antihistamine drugs seem to be antimetabolites of histamine. This opinion is not general among pharmacologists and biochemists. Therefore let us examine the facts in the matter and try to see the basis for this opinion and the objections to it.

Large numbers of compounds exist which will protect animals from shock caused by injection of histamine. Even more are known which will relax strips of smooth muscle (intestine or uterus) which have been made to contract by exposure to this same metabolite. Some of these agents will protect animals from certain types of anaphylactic shock caused by the injection of a protein antigen when the individual is sensitized to this same antigen. The reason so many of these drugs exist is the belief that histamine is responsible for many of the signs of an allergic response. The widespread clinical occurrence of allergies has stimulated attempts to control them pharmacologically.

Unequivocal proof of the functioning of histamine as the causative factor in anaphylaxis or in allergies is lacking. Nevertheless, a body

of circumstantial evidence amounting almost to proof has been built up over many years to indicate that histamine is liberated during the changes which follow the introduction of an antigen into a sensitized individual, and that this histamine is directly responsible for many of the allergic signs. Certainly exogenously administered histamine will elicit some of the responses, and the amounts necessary to do this are extremely small.

The first of the modern antihistamine drugs was discovered accidentally in 1933 by Fournau and Bovet (344) during a search for an antagonist to adrenaline. They found that thymoxyethyldimethyl-

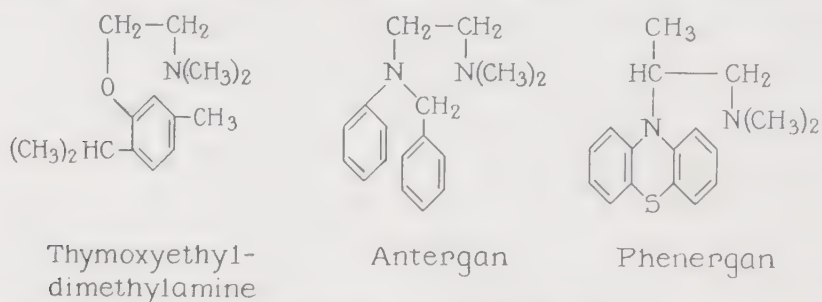


Figure 1. Structures of some important antihistamine drugs written to show their relation to histamine.

amine, the structure of which is shown in Figure 1, caused relaxation of strips of smooth muscle which had been contracted by application of histamine.

As more and more of such agents were uncovered by empirical testing, it became evident that nearly all were related in a greater or less degree to histamine (76, 118). This fact can be appreciated from the formulae of Figure 14 of Chapter 1, and of Figure 1 of this chapter. Since these drugs are structural relatives of the metabolite and since they antagonize the effects of either exogenous or endogenous histamine, it would seem logical to conclude that they act as antimetabolites of that substance.

This antagonism to histamine may be seen in a variety of test systems. Thus, the effects on isolated strips of smooth muscle of the drugs shown in Figure 1 are overcome by histamine in very small amounts. Likewise, strips of smooth muscle which have been taken from sensitized animals, and then caused to contract by exposure to the sensitizing antigen, are relaxed by application of the drugs. Most of the agents will protect animals from the shock which results when histamine is inhaled or injected. Some of them will save sensitized animals from anaphylactic shock which follows injection of the sensitizing antigen, but this property is not shared by all. The more active and least

toxic members of the series, such as pyribenzamine, benadryl, antergan, and phenergan, have found widespread application in clinical medicine (345).

The antagonism between histamine and the clinically active drugs just mentioned has also been demonstrated in the growth of three species of bacteria (469). The toxicity which the drugs showed to these microorganisms was overcome by additions of the metabolite just as has been found true with many recognized vitamins and their structural analogs.

In addition to its apparent role in causation of allergic responses, histamine also influences gastric secretion. An excess of it is held by some investigators to be the causative agent of gastric ulcers. This view finds support in the observation that injection of histamine into animals will eventually cause death from gastric perforation, provided that the shock which would otherwise kill the individual shortly after the injection is prevented by administration of one of the antihistamine drugs. Thus, these agents will not antagonize the effects of histamine on gastric secretion but will overcome many of the other actions of this metabolite. Attempts to make histamine analogs which will influence this other function of the metabolite are numerous, for obvious reasons. At least one substance has been shown to protect guinea pigs from the ulcers elicited by injection of histamine (346). This is N,N-dimethyl-N'-(2-pyridyl)-N'-(5-chloro-2-thenyl)-ethylenediamine.

Despite the antihistamine action of these drugs, which is undeniable, the structural analogy to histamine is not universally conceded. This is because more than one change is involved in passing from the metabolite to the drugs. The imidazole ring has been opened to yield an aliphatic chain,¹ and the side chain of the metabolite has been moved or eliminated. In some of the drugs, one of the original imidazole nitrogen atoms of histamine has been exchanged for an oxygen (e.g., thymoxyethyl dimethylamine and benadryl), and in all of them some alkylation of the nitrogen atom has been performed. These are a variety of changes, and they have been carried so far in some of the members of the series that many investigators are unable to see the relationship to histamine.² Especially is this so when it is pointed

¹ Antihistamine activity does not depend on this type of structural alteration. For example, triazolo derivatives of this metabolite have been prepared (347) and shown to possess antihistamine character. Here the ring system of the metabolite has been preserved but in a modified form by the exchange of carbon atom number 2 for a nitrogen atom in the analogs.

² The first clinically useful antihistamine agent was produced by examining a number of ethylenediamines. In the classical fashion, various ring systems were introduced usually in such a way that one of the ethylenediamine nitrogen atoms was

out that frequently the most active antimetabolites are those most nearly like the parent metabolite. In deciding whether this is a crucial point, the following considerations are noteworthy. If the imidazole ring of the metabolite were merely opened between positions 2 and 3, a highly active drug might be anticipated. However, this would leave a derivative of ethylenediamine with one of the amino groups unsubstituted. Probably it would be deaminated before it reached the site of action in the animal body. One can say that this amino group is alkylated, as it is in many antihistamine drugs, to prevent this destruction. Similarly, the removal of the side chain of histamine and the introduction of aromatic rings at positions 1 or 2 of the original imidazole ring may be viewed as maneuvers to retard destruction, to favor the transportation to the site of action, or to fix the drug to this site once it has arrived. These postulates have not been proved rigorously, but they do have some circumstantial support from empirical findings with other pharmacologic agents. The test of them will be possible when an isolated, cell-free system can be set up in which the functions of histamine can be demonstrated. In such a system, the most active compounds may be those most nearly related in structure to the metabolite.

Another objection which has been raised against the view that antihistamine drugs are to be regarded as antimetabolites of histamine is that substances have been found which antagonize the action of histamine and yet have no structural resemblance to it. This is cited as evidence that any structural similarity in this type of agent to histamine is probably coincidental. Because of the complex nature of the test systems in which antihistamine activity is measured, it is not possible to say whether the mode of action is the same for all effective compounds. However, in considering this objection one should remember that the enzyme histaminase shows antihistamine activity in animals or in organ preparations. This substance bears no known structural resemblance to histamine but, rather, acts by destroying it chemically. Conceivably other mechanisms as well might allow diverse substances to show an antihistamine effect. Although such considerations may seem to be pointing out the obvious, they are mentioned here because

a part of the ring. Since compounds of this type in which the heterocyclic ring was phenothiazine were highly active and of diminished toxicity (345), attention was centered on alkylated phenothiazines. The alkyl side chain was varied by lengthening it or by introducing methyl or ethyl branches. It is interesting to note that the best drug, namely phenergan, was the one in which the alkyl side chain approached the open form of an imidazole ring more nearly than it did in other compounds tested.

objections to inclusion of antihistamines as antimetabolites has been widespread and somewhat dependent on this argument.

Spasmolytic drugs

An explanation somewhat similar to that given for the antihistamine drugs may be extended to a group of the spasmolytic agents. These substances are able to overcome the spasms induced in strips of smooth muscle by application of acetylcholine or of certain non-specific agents.

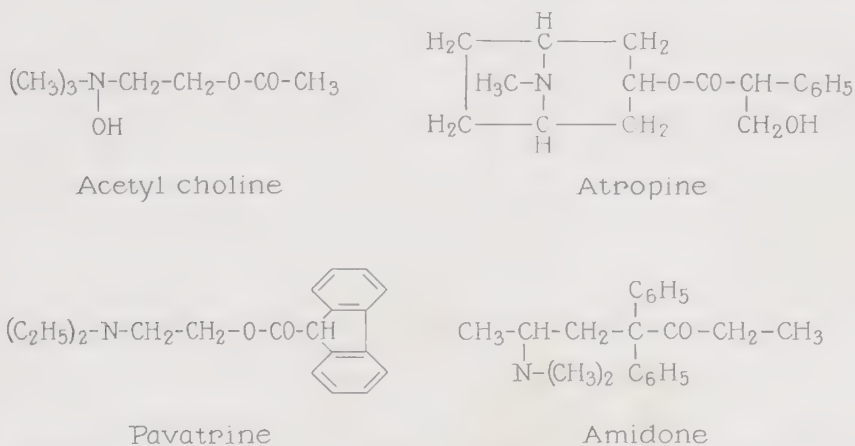


Figure 2. Structures of acetylcholine and of some spasmolytic drugs.

They have found use in therapy in attempts to control spastic conditions of the intestinal tract and of certain other smooth muscles. The first member of this series was atropine, a naturally occurring drug, the effects of which were discovered accidentally many years ago. As can be seen from Figure 2, it is an ester of tropic acid with a complex tertiary amine. Starting with this first member of the series, many attempts have been made to produce synthetically compounds with atropine action. Empirical testing of a great number of substances has revealed (332) that an alkylated ethanolamine or substituted ethanolamine is required and that this must be esterified through its alcoholic group to a derivative of acetic acid. In atropine, this acetic acid derivative is formed by replacement of one hydrogen atom with a phenyl group, and of a second hydrogen by a hydroxymethyl radical. In many of the synthetic spasmolytic drugs, a diphenylacetic acid has been used. In pavatrine, for example, diphenylacetic acid appears with the two phenyl groups joined by a carbon-to-carbon bridge. Most of the spasmolytic agents are tertiary amines, but the quaternary salts, made by full alkylation of the ethanolamine portion, likewise are effective. This is readily understood when one remembers the ease with which animals methylate tertiary amines and thus arrive at the quaternary

salts. Some of the members of this series of spasmolytic agents are shown in Figure 2. They may be regarded as acetylcholine in which the acetyl portion has been altered, and the choline can be present either as the quaternary salt or as the tertiary base. The structural analogy in all members of the series is not, however, very close. Especially is this true for atropine where the amine portion is quite dissimilar to choline. However, it is interesting to note that, as better spasmolytic drugs were produced as a result of empirical changes in atropine, they began to look more and more like acetylcholine. Thus, the amine portion most conducive to high activity proved to be dimethylethanolamine. The pharmacologic effects of most of these drugs on smooth muscle may be antagonized by acetylcholine. One of them (349) has also been shown to inhibit the action of cholinesterase of nerve tissue as this enzyme hydrolyzes the hormone (348). This is true of amidone, which is shown in Figure 2. The structural relationship of this drug to acetylcholine is somewhat different from that of other spasmolytic agents. Amidone is not an ester of an alkylated ethanolamine and a substituted acetic acid but rather may be viewed as having undergone a further alteration in which the oxygen atom of the ester linkage has been replaced by a carbon atom bearing two phenyl groups. A similar type of structural change is known among the orthodox antimetabolites and can be seen exemplified in phenyl-pantothenone and in β -acetylpyridine.

By exchanging the ester oxygen for a carbon atom in amidone, a drug with additional pharmacological properties was achieved. Not only does it have spasmolytic powers (349) but also it inhibits the enzymic action of cholinesterase and likewise possesses morphine-like properties. In fact, its morphine-like action has attracted so much attention that its other properties have not become well known. Neither the inhibition of cholinesterase nor the morphine action is prominent among other spasmolytic agents which are still esters.

The acquisition of new types of pharmacologic properties in a series of compounds with more or less gradual change in structure is not uncommon, and certainly suggests that the entire mode of action of a drug is not to be explained completely on just one basis. A single compound may hold within it features which relate it to two metabolites, or it may have resemblances to one and physical or chemical properties which modify its effects so that they are qualitatively not the same as those of other analogs of the same metabolite. Among accepted antimetabolites, one is reminded of α -tocopherol quinone which is related both to vitamin E and vitamin K, and while calling forth some of the signs of E deficiency it is counteracted by K.

The antagonism between acetylcholine and atropine has been known for a long time. It figured prominently in Clark's attempt in 1937 to explain drug antagonism on the basis of structural similarity of the contending substances (279). However, the idea that the relationship of the new synthetic members of the series to the metabolite lies in the acetic acid moiety, does not seem to have been widely held among pharmacologists.

The resemblance in structure of atropine and of amidone to acetylcholine is not great. The hormone is certainly nearer to synthetic spasmolytic agents, such as pavatrine, than it is to these drugs. It is surely debatable where the line must be drawn in order to include the obvious structural analogs and to exclude the compounds which are not antimetabolites. The acquisition of new features of pharmacological action as the structure of the drugs is changed more and more, complicates a decision. Nevertheless, some of the spasmolytic agents seem to be clearly, in some of their actions, antimetabolites of acetylcholine. Opinions differ about how many are. If amidone is to be considered among the antimetabolites, we must conclude that it interferes with several functions of acetylcholine. Not only does it antagonize the effects of the hormone on smooth muscle but it also inhibits the hydrolysis of it by cholinesterase. Atropine, on the other hand, seems to influence only the former of these functions.

Ideas about why some dissimilar drugs have similar pharmacologic actions

One of the puzzles of pharmacology is why two drugs which have quite dissimilar structures may have similar effects on an animal. This is one of the major difficulties in the path of any attempt to correlate structure and activity. Certainly substances which are superficially as unrelated as atropine and amidone would not be expected to have a similar effect. Some of these cases must be excluded from consideration because the causal relationship is quite indirect. For example, an ordinary esterase, if introduced at the proper point, might be quite antagonistic to acetylcholine because it would destroy it. Nevertheless, this protein would owe its antagonism to a cause quite superficial to that of the drugs just mentioned. A similar situation was discussed in the last paragraph of the section on antihistamine drugs.

If it were not known that atropine and amidone were each structurally related to acetylcholine, the dissimilarity in their constitutions would be so great as to make difficult the understanding of the similarities of their actions on muscles. Each drug is, however, related to this

hormone, and possibly owes a major part of its manifestations to the antimetabolite type of antagonism of it.

Among recognized antimetabolites we can see this situation clearly. For example, suppose that we observed that both pantoic hydrazide and salicylyl β -alanine inhibited microbial growth in competition with some unknown constituent of the medium. Without knowing that both drugs were analogs, each in a different way, of pantothenic acid and inhibited growth by causing pantothenic acid deficiency, we should be unable to understand why two such dissimilar substances exhibit the same type of action.

Ideas about why some closely related drugs may have quite dissimilar actions

Two antimetabolites may resemble each other and yet be related both structurally and functionally to two different metabolites. For example, 2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine and dihydroxyphenazine oxide (iodinin) are both phenazines and differ mainly in that the oxide nitrogens of the latter have been replaced by a hydrogen atom and a ribityl group in the former. The positions of the hydroxy groups in the latter substance are unknown but seem to be in the same ring as the amino substituents of the former compound. In addition, the former has two extra methyl groups attached to one of the benzene rings. As such variations go among antimetabolites, these are not great structural differences. We should, at first glance, expect both of these compounds, if they were drugs, to belong to the same series. Instead, however, we know that the one is an analog of riboflavin, and the other of vitamin K. Their basic actions in causing inhibition of microbial growth are quite different.

An even more striking example is to be found with methionine sulfoxide and methionine sulfoximine. These two compounds differ only in that the latter has an additional $=NH$ attached to the sulfur atom, and yet it has been established in bacterial growth experiments that methionine sulfoxide is an antimetabolite of glutamic acid, whereas methionine sulfoximine is such an agent for methionine. Casual inspection of the formulae of the two toxic substances would suggest that they are members of the same series, and yet, when their mode of action is investigated, they are found to belong to two quite different groups.³ With the realization of this, we should not be so perplexed

³ Although glutamic acid does not antagonize the action of methionine sulfoximine, a recent study (498) has shown that glutamine, as well as methionine, will overcome its effect on bacterial growth. Whether the antagonism was competitive was not demonstrated.

to find that two drugs which possess rather similar structures should act so differently as they sometimes do. The structural resemblance, as in these instances, may be purely coincidental.

The relationship to isosterism

Isosterism is a term used to describe the fact that two drugs which are related both in their pictorial chemical structures and in certain steric configurations may have the same or quite similar effects. Thus, in agreement with the steric or spatial resemblance of benzene and thiophene rings, two drugs which differ only in the replacement of a benzene by a thiophene ring system are frequently found to be quite alike pharmacologically. In like manner, thiazole and pyridine compounds should be isosteric and have been found to be so. This phenomenon of isosterism is an old one in pharmacology and has been examined extensively within the past two decades, especially by Erlenmeyer and his associates (350). Some surprise was, therefore, felt when the phenomenon of antimetabolites was discovered (351), because one of the first of them, viz., pyriethamine, should have been an isostere of thiamine. Indeed, it was first synthesized with the idea that it would show thiamine activity (3), as such an isostere should. The quite opposite action which this and succeeding antimetabolites showed was not compatible with the old ideas about the isosteric replacement among drugs.

This apparent contradiction of isosteres by antimetabolites is quite easy to resolve. Consider, for example, the sulfonamide drugs. Sulfapyridine and sulfathiazole are most certainly isosteric in structure and do show practically the same biological activity. On the other hand, sulfanilamide and *p*-aminobenzoic acid are not isosteres pharmacologically, even though they do fulfill the structural requirements of isosterism. Similarly, thiamine and pyriethamine have opposing actions biologically, whereas pyriethamine and the imidazole analog of thiamine are both antimetabolites of thiamine. By the old criterion, all three of these substances, because they are isosteric in structure, should have similar action. Obviously, the real explanation is that several similar drugs may have the same effect if all of them are derived from the same metabolite by related structural changes. The metabolite, however, will have an opposite action, antagonistic to the drugs, even though it may be isosteric with them. Pharmacological isosterism may be merely a phrase to describe the similar biological activity of a series of related analogs of one metabolite. In other words, isosteres could be groups of antimetabolites.

Antagonism between pairs of related drugs

With this view of similar effects of structurally related drugs, how are the antagonisms to be regarded which they sometimes show? At the outset it should be realized that there is no adequate explanation. Likewise, the existence of the antagonism is one of the serious challenges to the favored hypothesis about the mode of action of antimetabolites, if indeed such antagonisms do impinge on this field. However, some clarification of the issue may accrue from a marshaling of available facts, and the proposing of tentative ideas about their causation.

Two drugs may act antagonistically for diverse reasons. The one may stimulate a function which the other represses, or both may stimulate normal functions which are physiologically opposed to each other. In these effects, the antagonism of the drugs may be superficial or accidental and may imply no intimate metabolic relationship of them. Indeed, it is a safe assumption that two drugs, regardless of their character, may in some measure interact when they are given simultaneously to an animal (279). However, when the antagonistic agents are structural analogs which may be related to each other by exactly the same types of structural alterations which convert metabolites into antimetabolites, the case clearly falls in the realm of antagonism between structurally similar compounds which has been taken in this book as an index of the field of antimetabolites. The interrelationship may still prove to be accidental or casual, but, until this is shown, we must attempt to see in what manner it coincides with other manifestations of the antimetabolite phenomenon.

Salicylic acid and *p*-aminosalicylic acid are two drugs each of which will cause inhibition of growth of some types of microorganisms. The former seems to be particularly effective against those forms which synthesize their own supplies of pantothenic acid (247). The latter is peculiarly active against tubercle bacilli (144). This organism is, therefore, susceptible to both agents, since it is one of those species which makes pantothenic acid. When both drugs are tested together as inhibitors of the growth of tubercle bacilli, they are found to be antagonistic. The harmful effects of one of them can be largely overcome by proper amounts of the other, and, over a limited range of concentration, the antagonism approaches a competitive type. This can be seen from the data in Table 1, which have been taken from the work of Ivánovics (260). *p*-Aminosalicylic acid is an analog of the metabolite *p*-aminobenzoic acid and is derived from it by replacement of the hydrogen atom in position 2 by an hydroxyl group. The me-

Table 1

Antagonism of salicylic acid and of *p*-aminosalicylic acid in the growth of tubercle bacilli.
(The numbers represent percentage growth.)

(Reproduced with the kind permission of Dr. Ivánovics and the *Proc. Soc. Exptl. Biol. Med.*)

Salicylate, μ M	PAS, μ M								
	50	25	12.5	6.25	3.12	1.56	0.76	0.38	0.00
200	0	18	29	32	40	40	46	57	65
80	0	0	0	9	46	61	65	68	94
40	0	0	0	0	8	35	50	70	96
20	0	0	0	0	0	11	53	72	100
0	0	0	0	0	0	0	26	67	100

tabolite will antagonize the bacteriostatic effects of the analog, and in a competitive fashion at low concentrations of the drug. On the other hand, salicylic acid does not seem to be an antagonist to *p*-aminobenzoic acid. Here, therefore, are two drugs which interfere mutually and of which one, but not both, is clearly an antimetabolite.

The situation may be seen more clearly when we note that antagonisms between pairs of drugs, each of which is related to the same metabolite, have been found. For example, thienylalanine and naphthylalanine are both analogs of phenylalanine. When each is tested alone as an inhibitor of bacterial growth, only the thienylalanine competes with phenylalanine. The other analog is inactive. However, it is quite able to overcome the harmful effects of thienylalanine and in this action it is almost as potent as the metabolite (25). An obvious explanation of this drug antagonism is that the naphthylalanine can function in place of phenylalanine in a few of the essential reactions of this metabolite, but that it cannot fulfill all the needs. These same functions are the ones in which thienylalanine competes. Similarly, 5-nitouracil and 5-bromouracil, both analogs of thymine, mutually interfere in causing inhibition of growth of lactic acid bacteria (255). Possibly, this interference may also be understood in the manner just indicated.

Turning from drug antagonism in bacteria to the same situation in higher animals, we can see similar behavior. When evidence began to accumulate that curare owed much of its pharmacological effect to

an antagonism of acetylcholine at selected points in the nervous system (352), the idea of producing a simple synthetic compound related in structure to this metabolite and with curare-like action must have occurred to many. Whether or not this was the sole motivating force, drugs were soon produced which did have curare-form activity (353), and some of these were long aliphatic chains with quaternary ammonium groups at each end. One of the most active members was decamethylenetrimethylammonium chloride (398, 399). The relationship of this drug to acetylcholine may be said to be the replacement of the ester oxygen atom of the hormone by a methylene group and of the carbonyl oxygen by hydrogen atoms. Two such molecules have then been united tail to tail. This substance may be abbreviated C10. The corresponding pentamethylene compound C5 has only about 5 per cent of the curare action of C10. Small quantities of C5 or of curare will protect animals against the lethal effects of C10. The antagonism is qualitatively not complete because C5 does not influence the head-drop response caused by C10 or by curare, but merely interferes with the lethal action (354). Here again we see, as we did in the examples with bacteria, that it is the weakly active drug which antagonizes the more powerful one, and also that the antagonism may not be complete.

Although the preceding cases seemed complicated enough, we at least knew the metabolite to which the drugs bear structural relationship. Frequently this is not so. All that we then know is the antagonism between the closely related drugs. Consider morphine and allylnormorphine (259). These are two drugs which are quite similar in structure, but which counteract each other. When dogs have been treated with morphine, the resulting analgesia may be promptly abolished by allylnormorphine. This latter compound by itself has only weak analgesic effects.

In terms of the antimetabolite hypothesis, we would picture morphine as an analog of some metabolite, of which the structure is unknown. We would then be forced to consider that allylnormorphine could act in place of this metabolite for some of its functions. Such an explanation would be comparable to the one just outlined for the phenylalanine antagonists.

On the other hand, if we consider the hypothetical metabolite to have only one function, then the understanding of the experimental facts is even more difficult. Morphine would be pictured as displacing this metabolite, but, even if allylnormorphine then displaced the former drug, the site of action of the metabolite would still be occupied by the second foreign substance. The metabolite would still be excluded from its normal processes, and the animal should not recover.

The observed facts of antagonism between structurally related drugs usually show that one of the pair is much weaker than the other when they are tested singly. One of them may even be inactive when so tested. Furthermore, the antagonism usually is not perfect in that some manifestations of the one agent may not be completely overcome by the other. These features may possibly be understood in terms of the multiple functioning of the metabolite, as was discussed earlier in this section. However, this is only a suggestion of an explanation which has not been substantiated adequately experimentally. If this type of antagonism were understood for pairs of drugs related to known metabolites, the similar relationships between drugs not known to be derived from essential substances might be comprehended. The application of antimetabolites to this problem of pharmacology has not yet yielded anything but a feeling of kinship of unknowns, but, with this realization of the similarity of the situations involved, an intelligent way to attack the problem seems open. With this end in view, an investigation with the most simplified system (i.e., a cell-free one) of antimetabolites might be enlightening.

CHAPTER 9

Applications to biochemistry

Although we have been discussing more or less obliquely for the past eight chapters the applications of antimetabolites to biochemistry, several aspects remain. Let us consider these in the present chapter. To many investigators of biochemistry the antimetabolites have appeared to be rather subtle and useful tools with which to study mechanism of biological reactions. They have also offered promise, and some success, as aids for the discovery of new metabolites. The way in which they apparently block specifically one or two reactions has been especially appealing as a means of studying such processes in intact and otherwise normal individuals. As with all new tools, a considerable period is necessary in which the proper ways to use them, or at least the most profitable ways, are learned by experience. The past decade (1940–1950) probably represents a portion of such a period with the antimetabolites.

The discovery of new metabolites

The first new metabolite to be found through the use of an antimetabolite was *p*-aminobenzoic acid. The circumstances of this discovery have already been discussed. Before the demonstrations with sulfanilamide, this acid had not been widely encountered in cells, nor suspected of biological function. Soon after, however, it was isolated from yeast, and its presence detected in diverse living things. It was shown to be an essential growth factor for several species of microorganisms and within a few years was recognized as an integral part of folic acid. This has been one way in which new substances of biological importance have been discovered. Some of the others have

proceeded by application of the "favored hypothesis" of the mode of action of antimetabolites and those arising from it.

If an organism is retarded in its growth by an antimetabolite, one would expect that the metabolite would accumulate in the tissues, or that products of other reactions in which the metabolite is normally needed to carry through the usual metabolic steps would accumulate. These substances might be transformed slightly if they were so constituted as to allow their passage through relatively non-specific processes of oxidation, deamination, methylation, hydrolysis, or conjugation. If they were so transformed, easily recognizable derivatives of the new metabolites should appear. Thus, aside from these deriva-

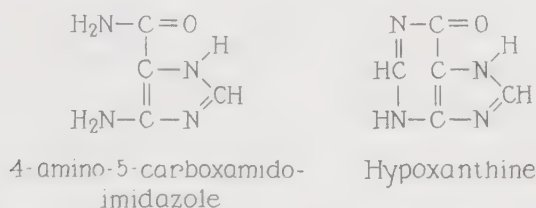


Figure 1. Hypoxanthine and its presumed precursor.

tives, either the structurally related metabolite or a new one which requires the participation of the former metabolite for its normal functioning should be detectable.

Studies with the analogs of *p*-aminobenzoic acid and of folic acid have led to the finding of 4-amino-5-carboxamidoimidazole as a metabolite which arises during the normal biosynthesis of hypoxanthine. Since some of the other biologically important purines can be made from this latter substance, the imidazole is presumably concerned with purine formation in general. The discovery of this new metabolite came about in the following way. When bacteria were grown in the presence of concentrations of sulfadiazine or sulfathiazole which were just sufficient to cause detectable retardation of growth, a diazotizable amine accumulated in the medium (367). This amine was not *p*-aminobenzoic acid, as one might expect if the sulfonamide drugs were interfering with the utilization of that metabolite, but rather it was found to be 4-amino-5-carboxamidoimidazole (325). As soon as it was identified, it was recognized to be related to hypoxanthine, because the insertion of a carbon atom between the amino and amido nitrogen atoms would yield this purine. This can be readily seen from Figure 1. A molecule of formic acid could react with the imidazole by elimination of two molecules of water in order to yield the purine. Such a reaction proceeds readily *in vitro*, as subsequent investigations showed (368). Some direct evidence was soon found

that a similar reaction might proceed in biological systems. Thus, radioactive formate added to liver homogenates appeared in position 2 of the purines which they formed (369), and radioactive 4-amino-5-carboxamido-imidazole in similar systems gave rise to radioactive hypoxanthine (459).

Part of the mode of thought which led directly to the identification of this new metabolite consisted of reflection on the non-competitive antagonism which exists between the sulfonamide drugs and the purines, such as adenine and hypoxanthine. We have already noted in Chapter 3, and elsewhere, that these purines were concluded to be products of reactions in which, at some point, *p*-aminobenzoic acid participated as substrate. The chain of events in the biosynthesis of hypoxanthine can now be visualized in the following manner. *p*-Aminobenzoic acid is converted, by a series of steps, into pteroylglutamic acid. This latter metabolite then functions in a series of reactions in which 4-amino-5-carboxamidoimidazole is caused to condense with the elements of formic acid in order to yield hypoxanthine or a derivative of it. This might then be aminated to give adenine, or oxidized to xanthine and to uric acid. If this scheme is correct, then a properly selected analog of pteroylglutamic acid should cause accumulation of this same imidazole in bacterial cultures, because sulfadiazine would be pictured as owing its similar ability merely to the creation of a lack of pteroylglutamic acid. Actually, when 4-amino-pteroylglutamic acid was used as the antagonist, the same imidazole precursor of hypoxanthine was found (370).¹ It would, therefore, appear that the purine precursor arises when some function of pteroylglutamic acid is interrupted, either directly through the use of one of its antimetabolites or indirectly through prevention of its synthesis by use of analogs of *p*-aminobenzoic acid. We shall see presently that other evidence has been found to indicate that pteroylglutamic acid is, indeed, involved in the biological formation of the purines and their conjugates, the nucleic acids.

¹ Inhibition analysis as it was described in Chapter 3 leads to the conclusion that *p*-aminobenzoic acid functions in several reactions. The one most sensitive to inhibition by sulfanilamide derivatives was indicated as that having to do with the formation of methionine. The next most sensitive was postulated as that concerned with the synthesis of the purines, and finally less sensitive processes such as those involved in the formation of pteroylglutamic acid were affected. The present results with 4-aminopteroylglutamic acid, however, tend to suggest that the sulfonamide drugs owe their effects primarily to inhibition of pteroylglutamic acid synthesis, and that processes resulting in the formation of purines and of methionine are affected by the primary lack of this metabolite.

After the discovery of 4-amino-5-carboxamidoimidazole, other evidence was soon found to substantiate the conclusions reached by the use of antimetabolites. For example, a mutant strain of a fungus was produced which required hypoxanthine as a growth factor. For this organism, the imidazole was able to replace the purine for promotion of growth (371). Nevertheless, some reservation about accepting this imidazole as the true precursor of the purines was felt, because many of the bacteria which produced it in the presence of suitable inhibitors would not convert it into hypoxanthine in the absence of those inhibitors. For this reason, the postulate arose that the imidazole was a transformation product of the true metabolite which accumulated when purine synthesis was inhibited. Some extraneous side reaction was viewed as changing the true metabolite into the imidazole actually isolated. Furthermore, there is some indication that hypoxanthine may not be the first stage in purine synthesis but rather that inosinic acid is formed and then gives rise to the free purine (460). Further work will quite probably clarify these details.

This example of the discovery of 4-amino-5-carboxamidoimidazole shows how the use of the antimetabolite phenomenon has uncovered a new metabolite. This compound accumulates when the functioning of a second metabolite is retarded by an antagonistic analog of it. In addition, the possibility is clear that some extraneous reaction may befall the true, new metabolite, so that the substance finally isolated is a transformation product of it. The chemical reactivity of many metabolic intermediates increases the probability of the latter occurrence.

A second means of discovering new metabolites through the use of antimetabolites is well illustrated by the work which showed thymidine to be an essential growth factor for several kinds of microorganisms. Methylfolic acid was added to a culture medium for the bacterium *Leuconostoc mesenteroides*, and then just enough folic acid to allow initiation of growth was included. When extracts of liver and certain other natural products were now added, a growth response was obtained. Although this effect could have been due to folic acid in the extracts, this was shown not to be the case. Rather, a crystalline substance which was considerably more active than folic acid was found to be the responsible agent. It was identified as thymidine (372). Inference would link this new compound to metabolic reactions involving the utilization of folic acid, just as similar deductions have been made about the role of *p*-aminobenzoic acid in relation to adenine and to methionine. Thymidine is the desoxyriboside of the pyrimidine thymine. Somewhat later it was learned that other desoxyribos-

ides of purines and pyrimidines likewise were active (373). Although thymidine had been known for some time in the laboratory as a partial hydrolysis product of nucleic acids, the fact that it might be a limiting factor in growth and metabolism in some species had not been established. The findings of its antagonism to an antimetabolite of folic acid suggested that it did so exist and function, and this was confirmed shortly thereafter by the observation that it and other desoxyribosides were essential growth factors for a variety of bacteria (373), i.e., that they were metabolites. From these observations some investigators have postulated that the formation of these desoxyribosides is one of the functions of folic acid.

Two early investigations had been carried out with antimetabolites in an attempt to find new compounds of metabolic importance, but both of these had turned up well-recognized metabolites instead. They, therefore, succeeded only in suggesting new aspects of the functions of old metabolites. The first was with sulfanilamide, and it led to the implication of *p*-aminobenzoic acid in the formation of purines and of methionine as we have already seen. The second was with phenylpantothenone (198). This analog of pantothenic acid inhibits the growth of yeast in a way which is not overcome by the vitamin. Addition of extracts of tissues to a yeast culture inhibited with phenylpantothenone allowed growth to occur. The substances in the extracts which were thus capable of antagonizing the action of the analog were identified as certain amino acids. Glutamic acid, aspartic acid, proline, or histidine was effective. Other amino acids were ineffectual. If the same argument were applied to this case as in the former ones, pantothenic acid would be implicated in reactions which yielded eventually these amino acids. Neither proof nor circumstantial evidence for this has appeared as yet.

Another way in which antimetabolites have been tested in efforts to discover new metabolites may be illustrated by the work with formylpteroylglutamic acid. Considering that the natural occurrence of formylpteronic acid in fungi such as *Rhizopus* was metabolically significant, and that a formic acid residue was required to convert 4-amino-5-carboxamidoimidazole into hypoxanthine, Gordon et al. (376) postulated that formylpteroylglutamic acid, in which the formyl group is attached to the nitrogen atom of the *p*-aminobenzoic acid residue, might be the functional form of folic acid. To test this hypothesis, *Streptococcus fecalis* R was inhibited in growth with 7-methylfolic acid, and the relative potency, as antagonists, of formylpteroylglutamic acid and of pteroylglutamic acid was examined. The formyl derivative was said to be about thirty times more powerful,

and this was taken as evidence for its existence as a natural metabolite derived from pteroylglutamic acid. In the absence of the inhibitor, formylpteroylglutamic acid was no more effective as a growth factor than was the unformylated vitamin. This evidence was insufficient to establish such a postulate, but it was valuable in the partial clarification of the functioning of folic acid. Some bacteria require as a growth factor a naturally occurring compound which they can synthesize, but only very inefficiently, from pteroylglutamic acid (461). Animals, also, synthesize this substance when they are fed pteroylglutamic acid. This new metabolite, known as folinic acid or citrovorum factor, is quite readily degraded to pteroylglutamic acid by exposure to a low pH. Folinic acid concentrates are very active in antagonizing the action of some analogs of folic acid which are less readily counteracted by this latter vitamin. Two means of testing are, therefore, available, one as an antagonist to folic acid analogs and the other as an essential growth factor for *Leuconostoc citrovorum*. The previous indications observed with formylpteroylglutamic acid aided in the synthetical formation of folinic acid (462). When formylated pteroylglutamic acid was hydrogenated in the presence of ascorbic acid, and the reaction mixture was heated, a substance with both kinds of folinic acid activity was produced. Its potency was great enough to suggest that the naturally occurring folinic acid had thus been formed. It was then found that direct hydrogenation of N-formylpteroylglutamic acid gave rise to a crystalline substance with empirical formula corresponding to N-formyltetrahydropteroylglutamic acid (478). Since this compound had the physical and chemical properties and the biological activity of concentrates of citrovorum factor (folinic acid), the natural metabolite was thus identified. A major part of this finding was the recognition of the biological activity of formylpteroylglutamic acid.

Thus far we have seen only examples in which not the metabolite analogous to the antimetabolite, but rather one metabolically connected with it, has been uncovered. Possibly the techniques involving antimetabolites are best suited to pick up these structurally dissimilar antagonists. If the related metabolite did accumulate as a result of being excluded from its normal reactions and were not decomposed beyond recognition by extraneous metabolic reactions in which it might be caught, the concentration of it frequently would be so low that it might escape detection by the crude procedures used thus far. For example, the amount of *p*-aminobenzoic acid which might be synthesized by a 1-liter culture of bacteria partially inhibited in growth by sulfanilamide would be of the order of 1 gamma. This value is arrived at by consideration of the requirements of those species of

microorganisms which need this metabolite supplied in the medium. The detection of such an amount of *p*-aminobenzoic acid in the presence of large amounts of sulfanilamide would be difficult with the chemical or biological means of estimation commonly employed.

However, eserine, an antagonist (possibly an antimetabolite) of acetylcholine, made possible one of the original isolations of this hormone. Eserine is well known to inhibit the action of cholinesterase. Since the enzyme is extraordinarily fast in its hydrolytic attack and is distributed in the tissues close to the site of action of the hormone, the preparation of tissue extracts in the usual way results in the complete destruction of the metabolite by the enzyme.² The isolation of the hormone from brain and other nerve tissue was accomplished by addition of eserine, which protected the metabolite from one of its enzymes (377).

The application of antimetabolites to these problems of biochemistry has yielded interesting new facts, but it is certainly not an exact science. Speculations of all sorts, many of which are possibly based on false assumptions, are guiding the attempts. As experience is multiplied, the selection of the proper manner of procedure and the interpretation of results will become more precise.

The attributing of new functions to previously recognized metabolites

Some of the ways in which new aspects of the metabolism of established metabolites have been found by use of antimetabolites have been indicated in the preceding section. Let us consider a few more examples.

A belief has slowly arisen that folic acid is concerned in the reactions which lead to the formation of purines and pyrimidines, and their derived substances, the nucleic acids. No unequivocal proof of this exists, but a body of evidence points to such a conclusion. Some of this evidence is that bacteria deficient in folic acid fail to form desoxyribonucleic acids in normal amounts (378). Another piece of evidence is that a mixture of adenine and thymine can take the place of folic acid in the nutrition of *Lactobacillus casei* and that when this occurs the organisms grow without containing detectable amounts of the vitamin (379). However, much of the evidence has arisen from studies with antimetabolites and then has been extended with other techniques. The participation of *p*-aminobenzoic acid in the syn-

² Other means of circumventing this hydrolysis have been the use of rapid refrigeration or precipitation with cold alcohol.

thesis of folic acid, as shown by investigation of the effects of sulfanilamide, taken together with the observation that purines will antagonize the bacteriostatic action of these antimetabolites, has implied to many that folic acid is involved in the synthesis of these purines. Analogs of thymine and of adenine have been made which inhibit bacterial growth, and which are antagonized in this effect by the related metabolites, or by folic acid, or by both (19). Thus, 5-bromouracil, a thymine analog, is antagonized in its effects on *Lactobacillus casei* by folic acid. Conversely, some of the analogs of folic acid, which compete with that vitamin, can also be antagonized by adenine plus thymine. Even the structural resemblance of the pteridine and purine ring systems is striking, because, by elimination of one carbon atom of the former, one arrives at the latter. This fact makes it difficult at times to say by casual observation whether a given substance is a derivative of folic acid or of guanine. Indeed, with some compounds designed as analogs of folic acid, such as 2-amino-4-hydroxy-6,7-diphenylpteridine the harmful effects can be overcome as well by the purine as by folic acid. It is as closely related in structure to one as to the other. Such facts, along with those previously discussed in this chapter, have played a prominent role in promoting the hypothesis that folic acid functions in purine metabolism.

Experiments with pantooyltaurine, an analog of pantothenic acid, first showed that this vitamin is taken up from the medium and conjugated into a more complex metabolite within the cell (180). When resting streptococci are suspended in pantothenate plus glucose and some appropriate source of amino acids, the vitamin disappears from the reaction mixture in the course of glycolysis. With pantooyltaurine as a specific inhibitor of the reaction involving pantothenate, the disappearance of the vitamin could be prevented without stopping the glycolysis. Without this breakdown of sugar, however, pantothenate was not metabolized, probably because no sufficient source of energy for the necessary synthesis was then available. Shortly thereafter, quite unrelated studies in enzymology showed that pantothenic acid did, indeed, exert much of its biological effect by prior conversion into a conjugate named coenzyme A (380). The biological synthesis of this coenzyme by yeast cells was then shown to be inhibited by antimetabolites of pantothenic acid such as phenylpantothenone. In the earlier experiments with streptococci, pantooyltaurine likewise probably behaved in the same fashion.

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The functioning of tryptophane as the biological precursor for the synthesis of nicotinic acid was established almost exclusively by classical nutritional experimentation, but early in the unfolding of this

discovery additional evidence was obtained by the use of antimetabolites (236). This evidence consisted of the demonstration that the pellagra-like manifestations called forth in mice by β -acetylpyridine were overcome not only by nicotinic acid but also by tryptophane. This finding helped in shifting attention to the metabolic conversion and away from the original hypothesis that bacteria contained in the intestinal tract of an animal and not the tissues themselves were responsible for the transformation. Considerable subsequent study showed that the tissues were, in fact, the site of the reaction.

Other obscure interrelationships among metabolites have been indicated by studies with structural analogs of one of them. These have occasionally been interpreted as suggesting that one compound arises from the other. For example, because the antibacterial action of β -hydroxyphenylalanine was overcome not only by phenylalanine but also in a non-competitive manner by tryptophane, this latter amino acid was said to be the precursor of phenylalanine (381). Whether or not this is true, the experimental finding would seem to suggest some metabolic connection between the two metabolites. When tyrosine was found to antagonize the action of thienylalanine, it was concluded that it was a product derived from phenylalanine (382). Such a change of phenylalanine into tyrosine seems quite plausible, and, indeed, has received support from experiments of quite different character. The relationship of phenylalanine and tryptophane is more obscure but has been supported by more recent shreds of circumstantial evidence from other types of experimentation. These illustrations will serve more to show ways in which these new biochemical tools, the antimetabolites, are being tested than to prove the certainty of conclusions which have been reached with them.

The tracing of metabolic pathways with antimetabolites

From the discussions in the previous sections it will be obvious that the use of antimetabolites has indicated the pathways of synthesis of some metabolites and has shown something of the intermediary compounds which are concerned in the processes. However, the very large gaps in the information so revealed stand out as well. A dozen or more steps may intervene between *p*-aminobenzoic acid and pteroylglutamic acid, or even between 4-amino-5-carboxamidoimidazole and hypoxanthine, and yet no indication of their existence or kind or number could be had from the means of handling antimetabolites now at our disposal. Similar shortcomings are found with other methods of tracing metabolic pathways, such as, for example, the use of mutant strains of

microorganisms (464). A variety of methods of study is needed in the investigation of this central problem of biochemistry.

The use of the antimetabolite idea in connection with other techniques has sometimes helped in the understanding of an intricate mechanism, as, for example, in the following case (300). For a mutant strain of the fungus *Neurospora*, either cytidine or uridine is required for growth. The growth-promoting activity of either of these pyrimidine nucleosides is antagonized by the purine nucleoside adenosine. At least two reactions in which adenosine acts as inhibitor could be distinguished. One of these was the deamination of cytidine to yield uridine. This is probably the reaction which explains why either of the pyrimidine nucleosides functions as a growth factor, because the one may be converted into the other by the organism. The deamination of cytidine was inhibited by adenosine, and this could be readily seen from the fact that addition of uridine to the inhibited culture allowed growth to proceed. In other words, when the product of the retarded reaction was supplied, the effect of the harmful agent was canceled. However, when more adenosine was present, the functioning of uridine also was inhibited and in a competitive fashion. Presumably, the incorporation of uridine into nucleic acid was then being prevented by the antagonist. On this inhibition, cytidine had no influence, presumably because its conversion to uridine was completely suppressed by the large amounts of adenosine. The harmful agent thus first overcame the conversion of cytidine to uridine and then, as the concentration was raised, began to retard the functioning of uridine. The metabolic pathway would thus seem to be the formation of cytidine, then its conversion to uridine, and finally the conjugation of the latter into more complex substances. Isolated enzyme systems will be needed in order to show whether these reactions actually occur. Even with such systems, the information derived from whole organisms will be required to understand what actually takes place.

Specific inhibitors of selected enzymes

The determination of the existence and nature of individual enzyme reactions has been the means by which most of our knowledge of the individual steps in metabolic reactions, and thereby the course of metabolism, have been found. The detection of an unsuspected enzyme among the host of such catalysts is difficult. Frequently its presence is first established by use of an inhibitor to which it is sensitive, while its neighbors which catalyze related reactions, are not. The value of such specific inhibitors is thus apparent, for, with them, new enzymic processes may be discovered. The poisons which have been

used in the past have usually not been antimetabolites but rather have been selective agents such as cyanide or fluoride which react chemically with specific parts of the enzyme system. However, the applicability of antimetabolites to this branch of biochemistry would seem obvious, and such compounds may find extensive use in the future.

First attempts have been made to obtain such agents for the proteolytic enzymes by application of the principles established with the antimetabolites. The structural requirements which must be realized in a peptide in order that it may be hydrolyzed by trypsin, chymotrypsin, carboxypeptidase, or pepsin are being established by trials of these enzymes on simple, synthetic peptides. The old studies of Bergmann and Fruton (383) and the more recent ones of Neurath and his associates (384, 385) have shown that chymotrypsin requires a substrate in which phenylalanine, tyrosine, tryptophane, or methionine is united through the amino group to an acyl radical, and through the carboxyl group as an ester or amide. When these structural requirements are met, hydrolysis occurs at the ester or amide linkage, and acylated phenylalanine, tyrosine, tryptophane, or methionine is set free. Enzymic action on a peptide such as glycyltyrosine amide is vigorous since this substance meets the structural requirements. The peptide bond involving glycine contributes to the ease of attack, and this is well shown by the fact that acetyltyrosine amide is only slowly hydrolyzed to acetyltyrosine even though it fulfills the other structural demands of the enzyme. If now acetyltyrosine amide is converted into an analog by replacement of the $-\text{NH}_2$ of the amide with $-\text{CH}_3$, a competitive inhibitor of hydrolysis of this amide is obtained (386). However, this substance 1-phenyl-2-acetaminobutanone-3 is but a weak antagonist as can be seen from the fact that it retards the action of the enzyme on acetyltyrosine amide but not on glycyltyrosine amide. Perhaps a more powerful inhibitor would be achieved if a phenyl rather than a methyl ketone were made. At least with analogs of pantothenic acid the formation of a phenyl ketone in place of the carboxyl group was much more successful than the introduction of a methyl ketone. The extension of experiments of this sort, making use of the recently acquired knowledge of structural requirements in the substrate, as well as of the means to convert a substrate into an inhibitor, may well lead to important discoveries among the proteolytic enzymes.

Investigation of the active centers of enzymes

Among enzymologists, an active center which confers on the particular protein its unique ability to attack specific substrates is believed to exist. The nature of this portion of the enzyme is unknown, except

perhaps in the iron-porphyrin compounds. Among dehydrogenases and decarboxylases, the old view was that the coenzymes, such as cozymase, flavin-adenine dinucleotide, thiamine pyrophosphate, and pyridoxal phosphate, were the active centers of the enzyme. Although this is partly true for the holoenzymes, we have come to recognize that the coenzymes are attached to the apoenzymes by certain active centers in the proteins. Indeed, even in the holoenzymes the specificity (i.e., the active center for the substrate) is contained within the protein portion. However, among the proteolytic enzymes, the esterases, the phosphatases, and numerous other enzymes, the nature of the active center is uncertain, and even its existence is doubted by some.

Attempts are being made to learn about this postulated active center by use of variously constituted substrates which are attacked by the enzyme, and by use of analogs of the substrate which inhibit this action. For example, investigation of the rates of hydrolysis of a great number of peptides by carboxypeptidase has revealed that this proteinase hydrolyzes those which contain tyrosine or phenylalanine with an unsubstituted carboxyl group, and with the amino group covered in peptide linkage by an acylated amino acid residue. The hydrolysis takes place in such a way as to liberate free tyrosine or phenylalanine, as the case may be. Carbobenzoxyglycylphenylalanine is thus one of the simplest such substrates. If the carboxyl group of such a substrate be combined as an ester, and thus obliterated, the enzyme does not attack it. The active center is, therefore, concluded to be such as to combine readily with a carboxyl group. More than this is involved however. Phenylalanine of the "unnatural" or *d*-series is a good inhibitor of carboxypeptidase, whereas the *l*-form, which occurs in proteins, is not. Since *l*-phenylalanine (or tyrosine) seemed necessary in the substrate at the carboxyl end of the peptide chain if hydrolysis was to occur, the participation of this structure in combination with the active center was indicated. Variation of the analogous inhibitor, namely, *d*-phenylalanine, showed that this was, indeed, most probable. Inhibition was strongest with β -phenylpropionic acid which lacked the amino group of *d*-phenylalanine but was in other respects the same. From this it was argued that the electrostatic effect of the amino group participated in the union of the substrate and active center. The importance of the carboxyl group could be seen from the failure of phenylethylamine to show any inhibitory activity. As the phenyl group was moved from a point in beta position to the carboxyl to either alpha or gamma, loss of inhibitory potency was found. From this finding again the need for a configuration in the active center to fit the β -phenylpropionic acid structure found in acylated phenyl-

alanine peptides was seen (290). Although such studies do not tell much of an exact chemical nature about the active center of the enzyme, they give an indication of it graphically which may aid in future attempts to define it. In the past, our fragmentary knowledge of the way in which a particular enzyme caused its effect was gained from a study of the rate at which it reacted with variants of the substrate. The relative powers of suitably constituted analogs of the substrate to inhibit reaction with it may tell as much or more.

Structural analogs of the substrate have been employed to begin the mapping of the active center of succinic dehydrogenase (387). This enzyme apparently owes some of its activity to the possession of $-SH$ groups, because it is poisoned by those reagents which react with such groups. The $-SH$ is apparently part of the active center, because, when the antimetabolite malonic acid is present in the system, poisons for $-SH$ groups do not destroy the enzyme. Presumably, these groups are protected by combination with the analog of the substrate. Malonic acid is specific in this respect, because it will not protect other $-SH$ -dependent enzymes from inactivation by reagents for thiols. An analogous situation has been shown with cholinesterase (388, 389). Diisopropyl fluorophosphate appears to react chemically with, and to destroy, the active esterase center. If eserine is present in the system when the phosphate is added, the enzyme is protected. Eserine has for many years been viewed by many investigators as a structural analog of acetylcholine even though the similarity is not great, and it has been known that it inhibits the action of cholinesterase. From these experiments the portion of the enzyme which combines with the substrate is concluded to contain a configuration which will react readily with diisopropyl fluorophosphate.

Localization of the role of various metabolites in morphological development by use of antimetabolites

One of the major problems of biochemistry is to relate the metabolic reactions observed *in vitro* to the understanding of physiologic processes. The biochemist would like to think that the chemical transformations of metabolites which he can study are the ones which play a part not only in the utilization of food but also in the morphological development of an organism. It is not easy to demonstrate this. Perhaps the most striking evidence for it is to be found in those experiments in which morphological changes in the development of an individual have resulted from the interference with the functioning of a single metabolite by the introduction of a structural analog of it.

When β -acetylpyridine is injected into the embryos of hens' eggs on the fourth day of development, a profound change in the morphology of the chicks is observed. If a sublethal dose has been employed, such embryos develop shortened and deformed limbs and other signs of morphological change (390). Since these changes are prevented by the administration of nicotinic acid along with the β -acetylpyridine, the production of them seems to be due to a failure of one or more reactions involving this vitamin.

Somewhat similar experiments have been done with analogs of folic acid. Injection of 4-aminopteroylglutamic acid into four-day-old embryos brought about profound stunting and abnormalities in the shape of the head, neck, beak, and legs (391). These gross abnormalities developed when the dose was adjusted so that immediate death did not occur, and the embryos continued to live for many days.

Because antimetabolites allow the creation of specific deficiencies at any stage of embryonic development, and also their termination at desired intervals, these agents seem to offer promise of some success in the study of the relationship of specific compounds to morphologic development. Perhaps they will aid in the understanding of the relationship of anatomical structure and metabolic function.

CHAPTER 10

The designing of antimetabolites

General types of structural alteration for converting metabolites into antimetabolites

From knowledge gained at first empirically it has been possible to discern and to formulate several general rules which indicate the types of structural change which will transform metabolites into antimetabolites. A few of these generalizations have been recognized for several years (31, 118, 205, 248), and new ones are being found among the hundreds of compounds which are tested for antimetabolite activity. These rules will not unfailingly predict the nature of new antimetabolites, but the chance that they will has been found very good. In fact, some of them have succeeded each time they have been tried. An antimetabolite predicted from these generalizations may not be active for a particular organism, but it is usually found to be an antagonist to the metabolite in some biological test system. This is to be expected from what is already known about the species spectrum of activity of such compounds. However, only very little is understood about the forecasting of the type of organism or tissue which a given variety of analog will affect. Nevertheless, an experienced investigator can frequently judge with some accuracy the type of structural alteration most likely to succeed in a given test system. Let us, therefore, examine some of these generalizations.

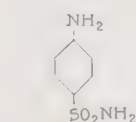
When they were first enunciated, some skepticism about their validity was felt because of the difficulties which usually have beset attempts to correlate chemical structure and biological activity. After they had proved useful in a variety of instances, their importance was questioned because it was said that they should be obvious from any

list of antimetabolites. However, since they were not always obvious in the beginning and since applications of them have been directly responsible for a considerable proportion of existing antimetabolites, they would seem to have some practical usefulness. In thinking about them, it should not be forgotten that they are empirical correlations and that exceptions can be found.

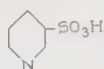
Replacement of carboxyl groups by other more or less acidic radicals

If the metabolite contains a carboxyl group, satisfactory antagonists to it can usually be made by exchanging this for some other more or

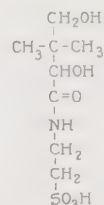
Class A



Sulfonilamide



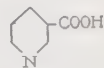
Pyridine-3-sulfonic acid



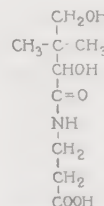
Thiopanic acid



p-Aminobenzoic acid



Nicotinic acid

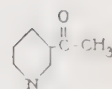


Pantothenic acid

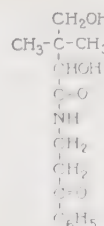
Class B



Amino-acetophenone



3-acetyl pyridine



Phenyl pantothenone

Figure 1. Antimetabolites derived from acid metabolites by exchanging carboxyl groups for sulfonic acid, sulfonamide, or ketonic radicals.

less acidic radical. A sulfonic acid or a sulfonamide, or an arsonic acid or amide, can thus be used. Likewise, the carboxyl group can be replaced by a ketone, or, in other words, an hydroxyl can be exchanged for an alkyl or aryl group. A few examples in which these modifications have succeeded are shown in Figure 1.

A proper degree of acidity in the new group seems to contribute to the success of an analog made in this way. For example, exchange of

Table 1

Dissociation constants and sulfanilamide type of activity of analogs of *p*-aminobenzoic acid measured on *Escherichia coli*

(Reproduced with the kind permission of Dr. Roblin and the *J. Am. Chem. Soc.*)

Num- ber	Compound	Acid Constants		$K_b \times 10^{12}$		Minimal Inhibi- tory Concen- tration, Molar \times 10^{-5}
		pK_a	K_a	1st	2nd	
1	<i>p</i> -Aminobenzoic acid	4.68	2.1×10^{-5}	2.6		
2	Sulfanilamide	10.43	3.7×10^{-11}	2.3		20.0
3	N ¹ -Methylsulfanilamide	10.77	1.7×10^{-11}	1.6		30.0
4	N ¹ ,N ¹ -Dimethylsulfanilamide	1.3		30.0
5	N ¹ -Hydroxyethylsulfanilamide	10.92	1.2×10^{-11}	2.0		50.0
6	Sulfanilylglycine	3.52	3.0×10^{-4}			>90.0
7	N ¹ -Phenylsulfanilamide	9.60	2.5×10^{-10}	1.4		3.0
8	N ¹ - <i>o</i> -Tolylsulfanilamide	9.96	1.1×10^{-10}	1.1		10.0
9	N ¹ - <i>m</i> -Tolylsulfanilamide	9.74	1.8×10^{-10}	1.3		5.0
10	N ¹ - <i>p</i> -Tolylsulfanilamide	9.82	1.5×10^{-10}	1.4		5.0
11	N ³ -Sulfanilylmetanilamide	8.23	5.9×10^{-9}	1.6		2.0
12	N ⁴ -Sulfanilylsulfanilamide	7.85	1.4×10^{-8}	0.8		0.5
13	N ¹ - <i>p</i> -Aminophenylsulfanilamide	10.22	0.6×10^{-10}	>10 ⁻⁹	0.7	5.0
14	N ¹ -Furfurylsulfanilamide	10.88	1.3×10^{-11}	1.8		20.00
15	Sulfapyridine	8.43	3.7×10^{-9}	3.8	0.1	0.6
16	3-Sulfanilamidopyridine	7.89	1.3×10^{-8}	10.0	0.4	0.2
17	2-S*-5-Bromopyridine	7.15	7.1×10^{-8}	0.8		0.5
18	5-S-2-Bromopyridine	7.12	7.6×10^{-8}	1.0		0.2
19	2-S-5-Aminopyridine	8.47	0.34×10^{-8}	10.0	0.3	0.6
20	5-S-2-Aminopyridine	8.82	0.15×10^{-8}	160.0	0.8	2.0
21	2-Sulfanilamidoimidazole	9.72	1.9×10^{-10}			40.0
22	3-Sulfanilamidopyridazine	7.06	0.87×10^{-7}	3.0	0.2	0.08
23	Sulfadiazine	6.48	3.3×10^{-7}	1.0		0.08
24	2-S-4-Methylpyrimidine	7.06	0.87×10^{-7}	1.2		0.2
25	2-S-4,6-Dimethylpyrimidine	7.37	0.43×10^{-7}	2.3		0.3
26	2-S-4-Aminopyrimidine	9.44	3.6×10^{-10}	13.5		20.0
27	4-S-Pyrimidine	6.17	6.7×10^{-7}	22.0	0.2	0.1
28	5-S-Pyrimidine	6.62	2.4×10^{-7}	0.8		0.2
29	5-S-2-Chloropyrimidine	5.80	1.6×10^{-6}			0.1
30	2-Sulfanilamidopyrazine	6.04	0.91×10^{-6}	0.6		0.08
31	4-S-1,2,4-Triazole	4.66	2.2×10^{-5}	0.7		>80.0
32	2-Sulfanilamidooxazole	6.5	3.2×10^{-7}			0.08
33	5-S-3-Methylisoxazole	4.2	6.3×10^{-5}			0.6
34	Sulfathiazole	7.12	7.6×10^{-8}	2.3		0.08
35	2-S-4-Methylthiazole	7.79	1.6×10^{-8}	2.3		0.2
36	3-S-4-Methylfuran	4.10	7.9×10^{-5}	0.8		1.0
37	3-S-5-Methyloxadiazole	4.40	4.0×10^{-5}	0.5		2.0

* S = Sulfanilamido.

TABLE 1 (Continued)

Dissociation constants and sulfanilamide type of activity of analogs of *p*-aminobenzoic acid measured on *Escherichia coli*

Number	Compound	Acid Constants		$K_b \times 10^{12}$		Minimal Inhibitory Concentration, Molar $\times 10^{-5}$
		pK_a	K_a	1st	2nd	
38	2-S-1,3,4-Thiadiazole	4.77	1.7×10^{-5}	1.4		0.6
39	2-S-5-Methylthiadiazole	5.45	3.5×10^{-6}	1.6		0.2
40	Sulfanilylcyanamide	2.92	1.2×10^{-3}			100.0
41	Sulfanilylurea	5.42	3.8×10^{-6}	0.6		10.0
42	Sulfanilylguanidine	5.6	0.03	10.0
43	Sulfanilylaminoguanidine	3.0	0.2	0.9
44	N ¹ -Acetylsulfanilamide	5.38	4.2×10^{-6}	0.6		0.7
45	N ¹ -Chloroacetylsulfanilamide	3.79	1.6×10^{-4}	0.4		10.0
46	N ¹ -Benzoylsulfanilamide	4.57	2.7×10^{-5}	0.6		0.3
47	N ¹ - <i>p</i> -Aminobenzoylsulfanilamide	5.20	6.3×10^{-6}	2.7	0.3	0.5
48	N ¹ -Ethylsulfonylsulfanilamide	3.10	7.9×10^{-4}	0.3		1,000.0
49	N ¹ -Sulfanilylsulfanilamide	2.89	1.3×10^{-3}			60.0
50	4,4'-Diaminodiphenylsulfone	3.1	0.2	2.0

a carboxyl group for a sulfonic acid radical would lead from *p*-aminobenzoic acid to sulfanilic acid, a compound which has very feeble, if any, bacteriostatic action. However, when a sulfonamide radical is used, sulfanilamide, which is relatively potent, is obtained. One can say that sulfanilic acid, a much stronger acid than *p*-aminobenzoic acid, is probably too acidic to be very active as an antagonist to this metabolite. Sulfanilamide, on the other hand, still retains acidic properties, since it is an aromatic sulfonamide, but it is not too strong. When one compares the antibacterial potency of various substituted amides of sulfanilic acid with their acidic strengths, one is even more struck by the changes in potency which differences in this property seem to engender. The data in Table 1 which have been taken from the work of Bell and Roblin (320) will illustrate this point. Maximal activity was associated with a pK_a near 6. Analogs which were stronger acids as well as those which were weaker were not as powerful. The most active derivatives were slightly less acidic than the metabolite itself. Only the ionized form seemed to be active, so that the derivatives which were most ionized at the pH at which testing was done showed the greatest activity.

Although such considerations of acid strength are important, they are not the sole criterion in deciding what type of analog to make.

For example, the halogenated sulfanilides of either pantothenic acid or of pimelic acid have proved to be better antimetabolites than the corresponding sulfonic acids or sulfonamides, even though acid strength would not have so indicated (314). Possibly, these halogenated substances are better because they may not be as readily displaced from combination with the enzyme by the related metabolites. Too much emphasis can be put on the relationship of acidic strength to potency, so that we should perhaps take note only of the possible existence of a causal influence.

The carboxyl group of the metabolite may be replaced by a ketone in order to achieve antimetabolites. When this is done one can discern a somewhat similar correlation of biological potency and of acidic strength (or at least of negativity) of the ketone. In passing from *p*-aminobenzoic acid to *p*-aminoacetophenone, or from nicotinic acid to β -acetylpyridine, the ketonic carbon is attached directly to an aromatic nucleus, which confers upon it some slight acidic character.¹ On the other hand, if an aliphatic acid such as pantothenic acid is converted to the corresponding methyl ketone, there is no aromatic nucleus to lend an acidic tendency to the compound. Actually, this analog of pantothenic acid showed no antimetabolite activity (169). However, if a phenyl, rather than a methyl, ketone was made, a powerful antimetabolite (phenylpantothenone) was obtained. Of course, this does not mean that pantothenic acid analogs must have an acidic character before they can function as antimetabolites. For example, pantoylpropylamine antagonizes the growth-promoting action of pantothenic acid in a few bacterial species and yet it has no acidic group. The comparison just referred to is concerned with ketonic analogs and does not seem to hold when a different type of structural relative is brought into the series. In the same way, comparison of acid strength of different types of analogs of *p*-aminobenzoic acid might show no correlation with potency, whereas in the sulfanilamide series the relationship is plain.

Replacement of one ring system in a metabolite by another

If the metabolite contains a ring system in its structure, and many of them do, the replacement of one atom in the ring by another has usually been found to yield antimetabolites. Single carbon atoms may be exchanged for nitrogen or for oxygen, or two carbons may be

¹ Although we may speak of a negative or acidic tendency in aromatic ketones, we should not forget that such compounds are not acids in the ordinary sense of the word. We cannot, for example, consider that β -acetylpyridine is at all similar in acidic strength to nicotinic acid.

replaced by a sulfur atom. Similarly, nitrogen atoms of the metabolite may be traded for carbons, or sulfur atoms for carbons or nitrogens. In this way, pyrimidine ring systems of the metabolite become benzene rings of the antimetabolite, or thiazoles become pyridines, or benzene structures become thiophene compounds. In like manner, naphthylenes may appear as coumarins. Examples of how this type of alteration has been applied are shown in Figure 2. One useful variation of this process is to replace an atom in the ring system of the metabolite by two hydrogen atoms, and thus to form open-chain compounds. For example, when the sulfur atom of biotin is thus eliminated, desthiobiotin, a rather potent antagonist to the vitamin, is obtained.

Since any one of several atoms in a ring system may be replaced and since each of them is not equivalent, a variety of antimetabolites may be formed by changes in one ring system. The activities of analogs so produced from a single metabolite may vary markedly. This can be seen rather clearly with derivatives of adenine. When nitrogens in positions 1 and 3 of the metabolite are replaced by carbons, 4-amino-benzimidazole is the result, whereas when carbon atom number 8 is exchanged for nitrogen, an aminotriazolopyrimidine is obtained. When these two analogs are tested on mice, or other mammals, a radical difference in their pharmacological effects can be seen. The benzimidazole brings about a flaccid state resembling in some respects anesthesia. The subjects can be placed at will in all kinds of grotesque positions where they will remain until the effects of the drug are dissipated. The triazolopyrimidine elicits no such response. In microorganisms, both analogs inhibit growth in competition with adenine, but the triazolopyrimidine is considerably more potent. Likewise, in the analogs of phenylalanine, the replacement of carbon atoms 2 and 3 of the phenyl ring by a sulfur atom gives a less potent inhibitor of bacterial growth than is obtained by the same exchange of carbons 3 and 4 (25). In other words, β -2-thienylalanine is less potent than β -3-thienylalanine.

Structural alterations of this type are not limited to ring compounds. For example, the sulfur atom of methionine may be replaced by an oxygen, or by a vinyl group, with the attendant production of compounds which have shown some antagonism to the related metabolite when they have been tested as inhibitors of bacterial growth (18, 25). Such findings may make the discussion of changes in ring systems seem artificial. Nevertheless, alterations of this type which have been made in open-chain compounds have thus far given rather weak antimetabolites. Probably there is nothing unique about ring systems in this

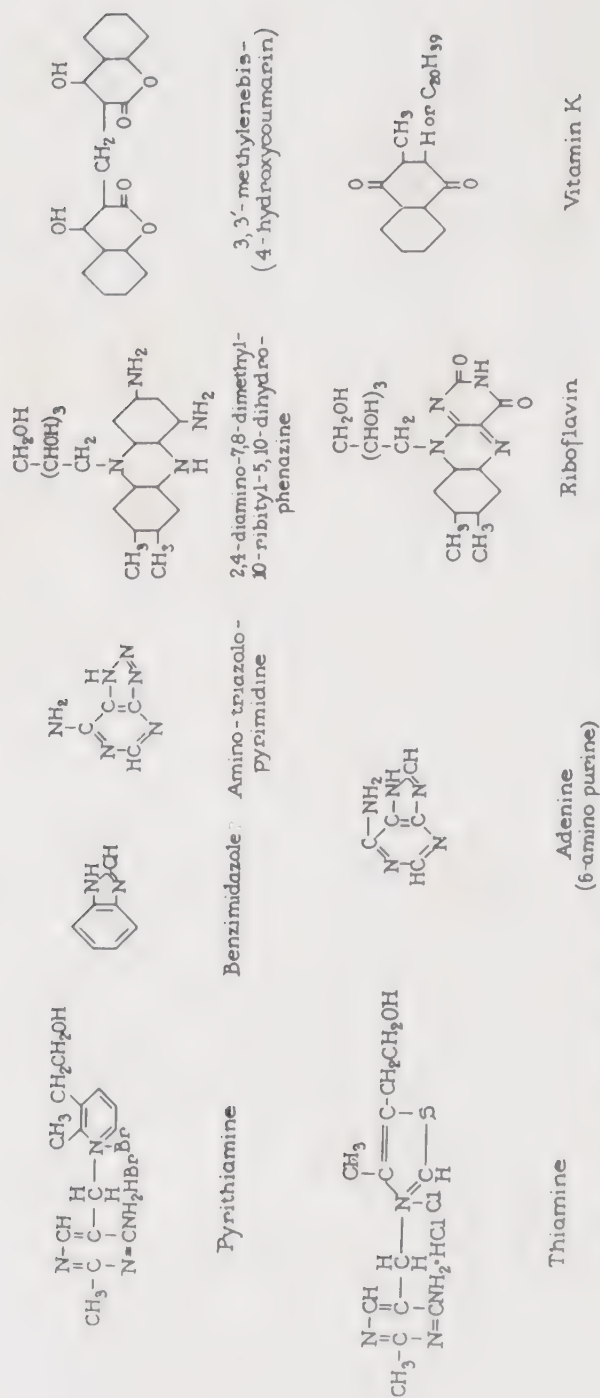


Figure 2. Examples of antimetabolites formed by exchanging ring systems in the metabolites.

respect, but, so far as our knowledge goes, they appear to stand apart for purposes of our present discussion. From a broad point of view, it is the exchange of one atom for another which in the long run is the only possible basis for formation of antimetabolites, whatever the nature of the metabolite may be.

The types of ring systems which have been used to replace those in the metabolites have usually been such as bear somewhat similar chemical reactivity. Thus, thiophene and benzene compounds have many properties in common, as do pyridines and benzenes. This consideration has been a factor in deciding which analogs to make according to this general method. Whether it has been a good reason is not known. It does not seem to be a requisite, because successful antimetabolites have been prepared by exchanging pyrimidine rings for benzene structures. Examples are benzimidazole (16) (antagonistic to adenine), the phenazine analog of riboflavin (109), and the quinoxaline derivative of folic acid (166). The differences in chemical behavior of pyrimidines and of benzenes are rather marked in some respects.

The exchange of alkyl side chains for halogens

When a metabolite contains an alkyl side chain attached to an aromatic ring system, the replacement of such a group by a halogen atom frequently yields an antimetabolite. Usually, too, it is of high potency and difficult to antagonize with the related metabolite. Some

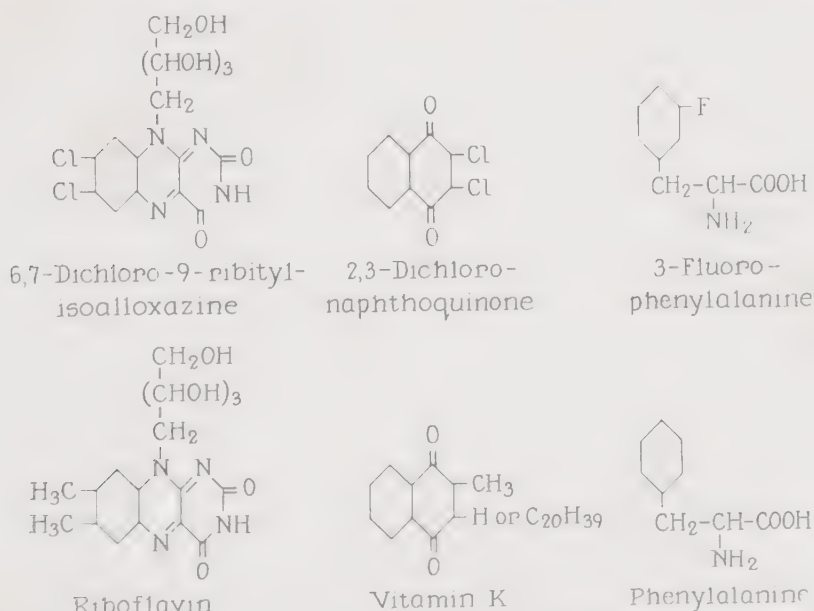


Figure 3. Antimetabolites formed by replacement of alkyl side chains with halogen atoms.

examples in which this type of change has been carried out are shown in Figure 3. The first member of this series, namely, the chlorine analog of riboflavin, was conceived by reflection on the similarity in crystal habit of compounds in which chlorine atoms replace methyl groups attached to benzene rings (105). The wisdom or generality of such a view is unknown but acquires interest in the light of alteration of crystal growth by isomorphic substances (see end of Chapter 3).

The choice of the proper halogen to substitute for a given alkyl group is influenced by considerations of relative size. Thus, if a methyl group is to be replaced, a chlorine atom seems to give a more potent antimetabolite than does a bromine atom. Methyl groups and chlorine atoms are said to occupy approximately the same space. Hydrogen atoms, which are possibly the smallest and simplest alkyl group, may be replaced by fluorine atoms in order to form potent antimetabolites. This is the type of alteration in passing from phenylalanine to fluoro-phenylalanine (355) or from acetic acid to fluoroacetic acid (13). In the latter case, only the fluoro analog, and not the chloro or bromo or iodo derivative, proved to be effective. Similarly, in the former case, 3-fluorophenylalanine was very active, whereas the corresponding chloro and bromo derivatives were at least 100 times less potent. With thymine, or 5-methyl-2,4-dioxypyrimidine, the replacement of the alkyl

Table 2

Potencies of various halogenated antimetabolites

Metabolite	Antimetabolite	Structural Change	Organism	Inhibition Index	Literature
Vitamin K	2,3-Dichloro-naphthoquinone	Cl for CH ₃ , Cl for H	<i>Saccharomyces cerevisiae</i>	0.1	208, 209
Riboflavin	Dichlororiboflavin	2Cl for 2CH ₃	<i>Streptobacterium plantarum</i>	0.25	105
Phenylalanine	3-Fluorophenylalanine	F for H	<i>Neurospora crassa</i>	1.5	355
	3-Chlorophenylalanine	Cl for H	<i>Neurospora crassa</i>	Greater than 150	355
	3-Bromophenylalanine	Br for H	<i>Neurospora crassa</i>	Greater than 150	355
Tyrosine	Monofluorotyrosine	F for H	<i>Neurospora crassa</i>	5	355
	Difluorotyrosine	2F for 2H	<i>Neurospora crassa</i>	Greater than 150	355

group with a bromine atom gave a good antagonist (356), but the corresponding chloro derivative was appreciably better.

The high degree of potency frequently found among these halogenated analogs can be seen from the data in Table 2. Furthermore, such compounds usually have the ability to affect organisms without regard to their nutritional requirements for the related metabolite. Finally, although competitive antagonism with the metabolite frequently can be observed over a narrow range of concentration, large amounts of the halogenated substances may exhibit a toxicity which is not overcome by the metabolite. This tendency is particularly marked among those derivatives in which the halogen atoms possess considerable chemical reactivity.

The exchange of amino groups for hydroxyls, or of hydroxyls for aminos

If the metabolite contains an hydroxyl group, the replacement of this by an amino group frequently leads to the formation of an antimetabolite. The reverse change also seems to succeed, for amino groups of the metabolite may be exchanged for hydroxyls. The chemical character of the hydroxyl group thus to be replaced does not seem to be crucial, for it may be phenolic, or carboxylic, or primary alcoholic in nature. For example, the powerful antagonist to folic acid, 4-aminopteroylglutamic acid, is the same as the metabolite except for the replacement of a somewhat phenolic hydroxyl in position 4 with an amino group. The reverse type of alteration in which a more or less phenolic —OH has been substituted for —NH_2 of the metabolite is to be seen in oxythiamine. This antithiamine differs from the vitamin only in this one respect (6). One of the antagonists to *p*-aminobenzoic acid is *p*-aminobenzamide which is achieved by substitution of an amino group for an hydroxyl of a carboxylic acid. Finally, the effect of exchanging alcoholic hydroxyls for primary amino groups can be observed with ϵ -hydroxy- α -aminocaproic acid. This substance arises when the primary amino group of lysine is removed and its place is taken by an hydroxyl. The compound so formed is very active in calling forth in rats the anemia and failure of growth which are seen in dietary deficiency of the metabolite (357). However, lysine will not overcome these manifestations.

As examples of this class of antimetabolites are multiplied one sees clearly that frequent difficulty is encountered in overcoming the action of the analogs with the metabolites. This may be manifested by lack of competitive antagonism, or by the lack of any demonstrable antagonism, as in the lysine analog. However, many of the antimetabolites

of this class do show competitive antagonism when small concentrations are tested or when the species of organism has been properly chosen. 4-Aminopteroylglutamic acid, for example, competes with folic acid in selected strains of lactic acid bacteria but is irreversibly toxic to most higher animals.

The replacement of amino groups by hydroxyls, or vice versa, does not always lead to an antimetabolite. For example, if the amino group in position 2 rather than the hydroxyl in position 4 of pteroylglutamic acid is so exchanged, an inert substance is formed (358). Likewise, pantothenic acid with an amino group in place of the secondary hydroxyl group is not an antimetabolite. The reasons for these failures are clear in several instances. For example, the amino analog of riboflavin in which the hydroxyl in position 4 of the isoalloxazine ring has been exchanged for —NH_2 is not an antagonist to the vitamin but, rather, shows riboflavin potency (360). This has been shown to be because the analog rather readily reacts chemically with water and hydrolyzes to yield the metabolite. Thus, if through either chemical or biochemical reactivity, an analog can decompose to yield the metabolite, or an inert substance, no antimetabolite activity would be expected. This may be only one reason for failure of this type of alteration in all circumstances.

Replacement of hydrogen atoms by hydroxyl or amino groups

Antimetabolites may also be made by exchanging hydrogen atoms for hydroxyl or amino groups. Some restraint is needed in this type of operation, for obviously hydrogen atoms are ubiquitous in organic molecules and antimetabolites do not seem to be. However, knowledge is still not extensive enough to say where the limits are and to predict what kinds of hydrogen atoms should be selected for such an exchange. This type of structural alteration has found most study among the amino acids, where an hydrogen on the β -carbon atom has been the one so replaced. Thus, β -hydroxyphenylalanine, β -hydroxyaspartic acid, β -hydroxyglutamic acid, and β -hydroxyalanine (or serine) have proved to be good antagonists to the related metabolites. The method is not limited to amino acids, however, because a potent antimetabolite of folic acid, namely, 2-amino-4,7-dihydroxy-pteridine-6-carboxyl-*p*-amino-benzoylglutamic acid, differs from the metabolite only in that an hydroxyl replaces hydrogen at position 7 and an oxygen takes the place of two hydrogens at position 9. The hydrogen atoms involved here give rise to phenolic and carbonyl functions rather than to secondary alcohols such as appeared in the former examples. From this we may conclude that the chemical reactivity of the hydroxyl groups

which thus are introduced is not crucial. This conclusion may also be appreciated from the fact that *p*-aminosalicylic acid, an antimetabolite of *p*-aminobenzoic acid, is derived by exchange of hydrogen for a phenolic —OH. A good example of the formation of antimetabolites by trading an hydrogen atom for an amino group is to be found with 2,6-diaminopurine, which is antagonistic to adenine (19).

Other types of structural change

The general types of structural alteration which have been described are not the only ways in which metabolites may be converted into antagonistic compounds. Consideration of the tables in Chapter I will show the variety of structural modification which have been found effective. For many types of alteration, however, too little information and too few examples are available to justify the creation of new classes. As more becomes known, more generalizations will be possible.

Steric and optical isomerism have occasionally been the only basis of difference between metabolite and antimetabolite, but up to the present time there have been only a few examples. The competition of *d*-(+)-leucine with *l*-(-)-leucine (81), and of *d*-(+)-tryptophane with the *l*-isomer (361), in the growth of lactic acid bacteria are cases in which biological antagonism of optical antipodes has been shown. Postulates have been made that the toxicity of certain antibiotic amino acid derivatives, such as penicillin, is attributable to their configurational relationship to the so-called unnatural *d* series of amino acids. Evidence in favor of this view is that the corresponding compound from the *l* series is not active (465). Just as with the optical isomers, antimetabolites arising from *cis-trans*-isomerism or from position isomerism have not been abundant.

This is not to say that optical configuration is of no importance among antimetabolites, because several examples are known which show clearly that the correct optical isomer must be employed if activity is to be observed. Thus, with pantooyltaurine only the enantiomorph with the same optical configuration as that of pantothenic acid shows activity, and the same relationship holds for other antagonists to this vitamin. This situation is somewhat surprising in view of the variety of structural changes which have been found to convert pantothenic acid into an antimetabolite. One might expect that a mere inversion of optical configuration would not, therefore, alter the biological properties so profoundly, especially since the optically active center can be dispensed with completely, as in salicylyl β -alanine with retention of good antimetabolite potency. The ability to combine with the specific proteins probably is the point at issue.

The various ways in which antimetabolites have been formed might suggest that any change in the structure of a metabolite is sufficient to yield an antagonist. This, however, does not seem to be true, because a considerable number of derivatives of a metabolite may show no antimetabolite potency, whereas some additional relatives do. This situation may be more apparent than real and may have arisen merely because the inert ones have not been tested in a sufficient number of organisms or other types of biological systems. Most certainly, an analog of a given metabolite may be inert for a number of systems, and yet, for the properly selected one, it may show antimetabolite activity. However, if one test system is employed, only certain types of derivatives show activity. Even then, as was discussed in preceding chapters, there is not one way, but several, in which antimetabolites can be formed from a single metabolite.

A combination of types of structural change may confer new properties or increased potency of the antagonistic compounds. A variety of examples have been discussed earlier, e.g., in connection with analogs of pantothenic acid and of folic acid.

Structural features associated with difficulty in demonstrating counter-action by the metabolite

Some types of structural alteration of a metabolite are frequently associated with the appearance in the antimetabolite either of a lack of competitive antagonism or of failure to demonstrate any reversal of effects at all. These structural features hold interest for several reasons. Firstly (see end of Chapter 6), this character in an antimetabolite usually is associated with a degree of potency far greater than that displayed by similar antimetabolites which lack it. Secondly, as might be expected if the analog were fixed at or near the site of action of the metabolite, a persistence and continuity of action not always found in competitive antagonists might accompany these difficultly reversible compounds. Finally, whenever nature herself has set out to make an antimetabolite, she usually has contrived to produce one which is difficult or impossible to antagonize with the related metabolite. Some of the reasons why this should be so seem rather clear, and these same reasons are often appealing to the biochemist who wishes to make an antimetabolite. One cannot say with finality what these types of structural alteration are, but accumulated experience points to some of the following factors.

When halogen atoms are introduced into a molecule of an antimetabolite, some difficulty is almost always experienced in demonstrating reversal of its effects by the related metabolite. This situation has just

been examined in one of the earlier sections of this chapter where it was pointed out that, although competitive antagonism might be shown over a limited range of concentration, irreversible toxicity usually appeared when larger amounts were tested. In fact, it would be hard to find a single instance in which competitive antagonism could be demonstrated over an hundredfold range of concentration. Usually, the competitive relationship, if it exists at all, becomes first non-competitive and then shades off into complete irreversibility as the concentration is raised. This is so, for example, with 2,3-dichloronaphthoquinone (209) when it is tested along with vitamin K in *Saccharomyces*. In other cases, the competitive type of antagonism may never be discernible, and, in order to show any ability of the metabolite to overcome the effects, a search must be made through many species until one is found which will allow demonstration of some sort of counteraction by the metabolite. It is thus with hexachlorocyclohexane. Chemical reactivity of the halogen atoms in such molecules may confer on them a toxicity unrelated to their antimetabolite effects, and this may explain their behavior. Living tissues contain enough amino groups and thiols which are essential to proper functioning so that alkylation or other change of some of them by an alkyl halide may cause irreversible injury. In one sense, this reaction would be non-specific. However, such an explanation would not suffice for those analogs which have halogens firmly bound in unreactive state to aromatic nuclei, but, among halogenated compounds, these have frequently proved to be the ones most readily antagonized by the related metabolites. Non-specific chemical reactivity of halogenated antimetabolites is probably not the only property which confers on them their unusual biological action. This can be appreciated from the fact that they show the ability to elicit the peculiar signs of deficiency of the metabolite to which they are related, even when they contain chemically reactive halogens. Perhaps their other resemblances to the metabolite attracts them principally to its site of action, where they then are attached securely either to this site or to neighboring molecules.

The effect of introducing halogen atoms can be readily seen from a comparison of the activities of sulfanilamide derivatives. The introduction of all sorts of substituents on the sulfonamide group serves only to affect the quantitative potency of the resulting compounds but does not influence the ability of *p*-aminobenzoic acid to compete with them. Thus, sulfapyridine, sulfathiazole, sulfadiazine, etc., vary in quantitative potency, but not in reversibility by the metabolite. When, however, the dibromo- or dichloroanilide derived from sulfanilamide is examined, two things stand out (362): (1) there is difficulty in dem-

onstrating competitive antagonism with the metabolite, and (2) the analogs have an unexpectedly high potency against microorganisms *in vitro*. This same difficulty in bringing about competitive counteraction and the same relatively high potency are found for the halogenated sulfanilides related to pantothenic acid and to pimelic acid (314).

A second way of forming antimetabolites which frequently leads to difficulty in demonstration of competitive antagonism is the replacement of hydroxyl groups with aminos, or vice versa. The difficulties experienced with these compounds are of the same kind as those mentioned for the halogenated analogs. This can be appreciated by remembering that 4-aminopteroylglutamic acid exhibits all gradations of reversibility by folic acid, from a competitive relationship with a few species of bacteria to failure to demonstrate counteraction in mammals. The poisonous properties for rats of ϵ -hydroxy- α -aminocaproic acid will also show that analogs which are completely irreversible in their effects may arise when an amino group of the metabolite is replaced by an hydroxyl (357).

The introduction of nitro groups into an antimetabolite frequently has this same type of effect. Although reduction to the amine may take place in the tissues, thus bringing this class into the same category as the amino analogs of hydroxy compounds, such reduction does not always take place. We must, therefore, attribute a separate effect to the nitro group. For example, with chloromycetin the nitro group seems to remain unaltered by many living things, and yet the introduction of this group into various phenylalanine analogs, including chloromycetin, is associated with the appearance of difficulty in demonstrating competitive antagonism over a wide range of concentration.

A third consideration is not well illustrated by studies of graded series of compounds, but it is that the greater the structural change has been in passing from the metabolite to the analog, the more likelihood of difficulty there will be in demonstration of antagonism by the metabolite. Analogs, such as 3,3'-methylenebis-(4-hydroxycoumarin) and γ -hydroxybutyryltaurine, which differ from the metabolites (namely, vitamin K and pantothenic acid) by several distinct structural alterations rather than by a few are often only poorly antagonized by the metabolite and may be completely irreversible. A tendency in this direction with progressive alterations in phenylalanine can be seen from the discussion in Chapter 5 of chloromycetin and this amino acid. (See Table 3 of Chapter 5.) This difficulty in demonstrating antagonism may be only a reflection of the fact that these analogs are so different from the parent metabolite that their toxicity is due in part

to causes other than an antimetabolite effect. On the other hand, it may arise from the causes discussed in Chapters 2 and 3 in the sections on non-competitive antagonism.

Apparent relationship between potency of an antimetabolite and the magnitude of the structural change in arriving at it

If one wishes to make an highly active antimetabolite, is it better to alter the structure of the metabolite as little as possible, or to a rather great extent? Before this question can be discussed intelligently, two considerations should be noted. In the first place, as just discussed, difficulty in counteracting the effects of an antimetabolite sometimes is associated with a high degree of structural change, and this difficulty frequently accompanies high potency. Therefore, in order to simplify the quest, comparisons should be made among groups of analogs in which each individual exhibits antagonism of the *competitive* kind. In the second place, no adequate criterion of the magnitude of a structural change exists. Is the passage from a benzene to a pyridine compound less of a change than the jump from benzene to pyrimidine? Since in the former only one carbon atom has been replaced by a nitrogen, instead of two as in the latter, the alteration would seem less, but this pictorial way of deciding the issue may not be the correct one. In fact, the pictorial method can be criticized as Rydon has done (466) as being too superficial. Some advantage has been shown, as in the methyl analogs of tryptophane, from consideration of electronic configuration and of actual intramolecular distances between hypothetical points of attachment of the metabolite to its enzyme. By placing the methyl group in the position calculated from these considerations, a more active inhibitor of bacterial growth was achieved than when the same structural alteration was made at other points. However, until we have an adequate measure of the similarities of compounds, the pictorial method must be our reference, unless obviously it should be modified in individual cases by sufficient knowledge of the physical and chemical behavior of the substances under examination.

With these considerations in mind, it is usually clear that a small change results in a more active antimetabolite. Among the easily reversible analogs of folic acid, for example, pteroylaspartic acid is more active both in higher animals and in bacteria than is 2-amino-4,7-dihydroxypteridine-6-carboxylyl-*p*-aminobenzoylglutamic acid, and this in turn is better than quinoxaline-2-carboxylyl-*p*-aminobenzoylglutamic acid. The first of these substances differs from the metabolite only in lacking a $-\text{CH}_2$ group in a long alkyl side chain. The second has an hydroxyl group in place of the hydrogen atom at position 7,

and also a —C:O— radical instead of the methylene bridge between the ring systems of folic acid. The third has this latter alteration plus the loss of amino and hydroxyl substituents of the pteridine ring, and also the replacement of the two nitrogen atoms in the pyrimidine portion of the pteridine ring system by carbon atoms. The relative potencies of these three analogs as inhibitors of the growth of *Lactobacillus casei* are in the ratio of 1:3.5:150 (166).

Similarly, among competitive antagonists of pantothenic acid, β -methylpantothenic acid, which differs from the vitamin only in having one hydrogen atom replaced by a methyl group, is more active than is pantoylpropylamine, which has —CH_3 instead of —COOH (see Table 6 of Chapter 1). Similar relationships have been noted in enzyme systems, where, for example, it was found that of the alkyl derivatives of succinic acid the most active inhibitors of succinic dehydrogenase were the ones with smallest alkyl groups (see Chapter 4). These are the ones which are nearer in structure to the metabolite.

Apparent contradiction of the above-mentioned generalization is found with a few analogs rather distantly related to metabolites, which instead of being antagonistic have weak metabolite potency. One would expect these distant relatives either to be inactive or weak competitors and would anticipate that any analog with metabolite potency would be very close in structure to the metabolite itself. The contradictions are, therefore, worthy of note. For example, thiazole-5-sulfonic acid is said to be weakly active as nicotinic acid for *Staphylococcus aureus* (95), whereas pyridine-3-sulfonic acid (or amide) and thiazole-5-carboxamide are antimetabolites of nicotinamide. These two latter analogs exhibit singly the structural alterations which together go to make up thiazole-5-sulfonic acid. Likewise, the quinazoline analog, N-[*p*-(4-quinazoly)benzoyl]glutamic acid, of pteroylglutamic acid is said to show weak folic acid activity for *Streptococcus fecalis* (363), whereas the antimetabolites mentioned in the preceding paragraph, which differ less in a pictorial sense from the metabolite, are antagonists. The slight metabolite activity of these two substances has not been studied in any detail, and it may be that it does not represent true vitamin effect. The large amounts of the thiazole sulfonic acid, for example, which are needed for a demonstration of nicotinic acid potency may be a source of nutrient on which the organism can grow without the intervention of nicotinic acid, just as it has been shown that certain bacteria can grow on amino acids rather than on the usual glucose substrate, without the aid of this vitamin (364). However, it is well to remember that, so far as the published reports go in describing the

metabolite potency of these rather distant structural analogs, they speak against the generalization under discussion.

At first glance, the derivatives of sulfanilamide, likewise, would seem to contradict the generalization. Thus, sulfanilamide looks rather more like *p*-aminobenzoic acid than does sulfathiazole, and yet the latter analog is the more active in causing inhibition of bacterial growth. We have already seen, however, that this situation is to be understood, at least in part, on the basis of differences in acid strength of the two antimetabolites. Perhaps, if both compounds penetrated to the site of action equally well, sulfanilamide would be the more potent. Evidence for this view is that by proper selection of a *pH* at which to do the test, the difference in activity disappears. Considerations of this sort must be kept in mind both in the application of the generalization and in the critique of its truth.

Epilogue

The epilogue of Chapter 2 could be repeated here with equal propriety. In addition, it should be said that the principles of this present chapter are the ones which have been found useful in the early explorations of the antimetabolite phenomenon. A network of postulation from insufficient data has of necessity surmounted the structure, but in looking at the development of biochemistry it can be realized that the normal process is the erection of hypotheses, most of them aerial and ethereal. The well-rooted ones grow into the very framework of the science by the gradual accretion of data. Some become moribund and must be replaced with structures which frequently look rather similar, even if they are not identical with the original part which failed.

CHAPTER 11

Practical suggestions for the synthesis and testing of antimetabolites

Since biochemistry is an experimental science, this monograph should be concluded with the detailed description of some actual experiments. These have been chosen to illustrate some of the features discussed in general terms in preceding chapters. Most of the demonstrations have an historical importance which has recommended them from among the large number of similar ones which might have been chosen. The description of the methods of synthesis of the compounds used in the biological trials has been prompted by the inaccessibility of these substances to most investigators, even at the present time. Therefore, an effort has been made to begin each synthesis with readily available materials.

Synthesis of pyrithiamine

An impure preparation of pyrithiamine was first described by Tracy and Elderfield (3). The last step in their procedure was improved by Wilson and Harris (4) who were then able to isolate a pure compound to which they gave the name neopyrithiamine. The biological effects of the older preparation have been shown to be due to its content of the pure substance of Wilson and Harris (467). Therefore, their modified procedure will be presented here. A few other improvements in some of the stages have also been included. The reactions are illustrated by the formulation in Figure 1.

Ethyl- α -(β -ethoxyethyl)-acetoacetate. One hundred and eighty grams of ethyl cellosolve were mixed cautiously with 542 grams of PBr_3 , and the mixture was refluxed for 4 hours. It was then cooled and treated with 400 cubic centimeters of ice and 500 cubic centimeters

of ether. The ether layer was dried and distilled through a fractionating column. The ethoxyethylbromide was then collected as 140 grams of liquid which boiled at 126–128°.

In a mixture of 700 cubic centimeters of dioxane (previously distilled from sodium) and 156 grams of ethyl acetoacetate, 28 grams of sodium were dissolved. This solution was heated to boiling and slowly treated with 194 grams of ethoxyethyl bromide. The resulting mixture was stirred and heated under reflux on a steam bath for 21 hours. It was

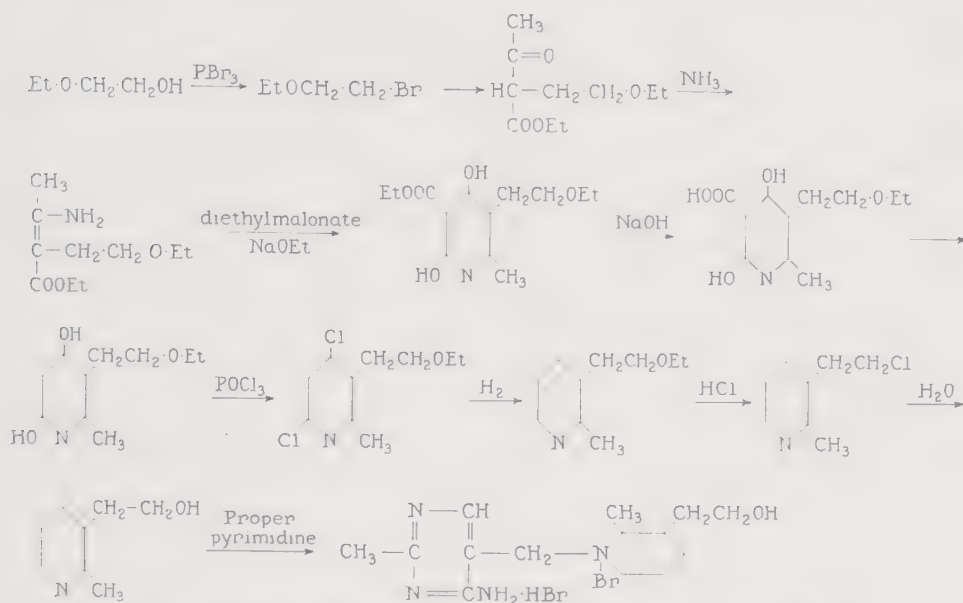


Figure 1. Reactions involved in the synthesis of pyriethamine.

then cooled, neutralized with glacial acetic acid, filtered, and washed with dioxane. The filtrate was then distilled under reduced pressure. Fraction boiling at 117° at 11 millimeters pressure was collected and was found to weigh 94 grams.

Ethyl- α -ethoxyethyl- β -aminocrotonate. A mixture of 31 grams of ammonium nitrate and 94 grams of ethyl- α -(β -ethoxyethyl)-acetoacetate was saturated with dry NH_3 at 0°, and then heated in a closed pressure bottle at 65° for 7 hours. The temperature was raised slowly during the first half of the reaction period. The bottle was opened, the contents were mixed with ether and water, and the ethereal solution was dried with magnesium sulfate. The ether was then removed under reduced pressure, and low-boiling impurities were removed by distillation until the boiling point reached 100° at 11 millimeters pressure. The liquid remaining in the distilling flask was then used for the next reaction. It could be purified further by distillation at 0.4 millimeter pressure.

Under such conditions its boiling point was 96.5–98.5°. The substance melted at 13–14° and decomposed slowly on standing at room temperature.

2-Methyl-3-ethoxyethyl-4,6-dihydroxy-5-carbethoxypyridine. To a solution of 9.2 grams of sodium in 175 cubic centimeters of absolute alcohol were added 64 grams of diethyl malonate, and then 0.4 mole of ethyl- α -ethoxyethyl- β -aminocrotonate. The mixture was heated under reflux in an oil bath at 140° for 21 hours. The reaction mixture was then concentrated slightly under reduced pressure and treated with 1 liter of ether. The precipitate of the sodium salt of the desired pyridine was filtered off and washed with ether. It was then dissolved in water and filtered through a wet paper in order to remove traces of malonic ester. When the filtrate was adjusted to pH 5.5 with HCl, a bulky precipitate of the desired pyridine was produced. This was collected on a filter, washed with water, and dried. The yield was 35 grams. When recrystallized from alcohol, the compound melted at 174–176°. The substance was soluble in either dilute alkali or dilute acid.

2-Methyl-3-ethoxyethyl-4,6-dihydroxypyridine. A solution of 35 grams of 2-methyl-3-ethoxyethyl-4,6-dihydroxy-5-carbethoxypyridine, in 200 cubic centimeters of 10 per cent aqueous NaOH, was refluxed for 3 hours. While this solution was still hot it was made acid to thymol-blue with concentrated HCl and was then adjusted to pH 5.5 by addition of solid sodium acetate. While the mixture was acid a considerable evolution of carbon dioxide occurred. After addition of the sodium acetate the mixture was heated on the steam bath for an hour and was then cooled and filtered. The product was washed with water and dried. The substance could be purified for analysis by recrystallization from dilute alcohol. After such treatment it melted at 290–293°.

2-Methyl-3-ethoxyethyl-4,6-dichloropyridine. A mixture of 25 grams of 2-methyl-3-ethoxyethyl-4,6-dihydroxypyridine, 150 grams of POCl₃, and 10 grams of PCl₅ was refluxed for 6 hours. Excess POCl₃ was removed by distillation under reduced pressure on a water bath. Water was added to the residue, and the solution was made strongly alkaline with KOH and then extracted continuously with ether overnight. The extract was dried with magnesium sulfate and distilled under reduced pressure. It boiled at 98–99° at 0.4 millimeter. The product was a solid which melted at temperatures slightly above those of the room. The yield was 4 grams.

Unchanged starting material was recovered by neutralization of the aqueous solution after the ether extraction. The precipitate which was

thus collected was returned to the process when the next batch was prepared.

2-Methyl-3-ethoxyethylpyridine. A solution of 15.2 grams of 2-methyl-3-ethoxyethyl-4,6-dichloropyridine and 6.35 grams of potassium acetate in methanol was shaken with 1 gram of palladium black (American Platinum Works) in an atmosphere of hydrogen at normal pressure. After 4 hours, 3,375 cubic centimeters of wet hydrogen at 25° and 733 millimeters had been absorbed; calculated for 2 moles of wet hydrogen: 3,390 cubic centimeters. The filtrate from the catalyst was concentrated to dryness under reduced pressure after the addition of 5 cubic centimeters of concentrated hydrochloric acid. The residue was dissolved in the minimal amount of water, the solution was saturated with potassium hydroxide, and then extracted with 8 portions of ether. After drying the ethereal solution, first with solid potassium hydroxide and then with sodium, the pyridine was distilled from sodium at reduced pressure. The yield of material boiling at 72–73° at 0.5 millimeter was 9.1 grams or 81 per cent.

The picrate crystallized as fine, lemon-yellow needles on addition of about 5 volumes of ether to its concentrated alcoholic solution and melted at 63–64°.

The chloroplatinate crystallized as orange tablets from alcohol containing a trace of hydrochloric acid. It melted with decomposition at 165–168°.

2-Methyl-3-chloroethylpyridine. A solution of 4.2 grams of 2-methyl-3-ethoxyethylpyridine in 35 cubic centimeters of concentrated hydrochloric acid was heated in a sealed tube at 150° for 3 hours. The contents of the tube were decolorized with carbon (Norit) and then concentrated to dryness under reduced pressure several times with water. Portions of the crystalline residue were removed for the preparation of derivatives, and the main amount was hydrolyzed further as indicated below.

The picrate crystallized as silky, lemon-yellow needles from alcohol and melted at 134–135°.

The chloroplatinate formed stout, orange needles or prisms from water acidulated with hydrochloric acid and melted with decomposition at 189–190°.

The chloraurate was recrystallized by careful dilution of its cold alcoholic solution. It formed stout, lemon-yellow prisms and melted at 116–117°.

2-Methyl-3-hydroxyethylpyridine. The above chloroethylpyridine hydrochloride was heated with 50 cubic centimeters of water in a sealed tube at 160° for 4 hours. The contents of the tube were decolorized

with carbon (Norit) and concentrated to about 10 cubic centimeters under reduced pressure. The solution was then saturated with potassium hydroxide and exhaustively extracted with chloroform. The combined chloroform extracts were dried with magnesium sulfate and concentrated to about 5 cubic centimeters. On addition of petroleum ether (Skellysolve B), the pyridine crystallized as the monohydrate after scratching and chilling. It was recrystallized from chloroform-petroleum ether and formed stout prisms which melted at 61–62°. The hydrate was unusually stable and distilled unchanged at 120–125° at 0.5 millimeter. It was sparingly soluble in ether, although freely soluble in chloroform and methyl and ethyl alcohol.

The picrate formed fine needles from alcohol ether and melted at 123–124°.

Pyrithiamine. In order to obtain pure pyrithiamine it was necessary to use an excess of the pyridine reactant and to conduct the condensation at ordinary temperatures. The pyrimidine reactant is used in the commercial synthesis of thiamine and is thus readily available. First 600 milligrams of 2-methyl-3-hydroxyethylpyridine were dissolved in 3–4 cubic centimeters of isopropyl alcohol. Then 240 milligrams of 2-methyl-5-bromomethyl-6-aminopyrimidine dihydrobromide were added, and the mixture was shaken until solution was obtained. The resulting solution was filtered and was allowed to stand overnight at room temperature, during which time the product separated. It was centrifuged, washed with fresh isopropyl alcohol, then with petroleum ether, and dried. The yield of pyrithiamine hydrobromide was 200 milligrams (73 per cent based on pyrimidine); melting point was 205–210° with decomposition. The melting point was not very characteristic and differed considerably for individual samples which were analytically pure.

Synthesis of phenylpantothenone

Synthesis of phenylpantothenone may be accomplished readily by following a method described in the literature (169). It is desirable to adhere strictly to the conditions recommended there, because when variations are introduced into the final step the great reactivity of the free amino ketone may contribute to the formation of impure products. For example, this free amino ketone will condense with itself.

β -Aminoethylphenyl ketone hydrochloride. A mixture of 18 grams of β -alanine and 30 grams of phthalic anhydride was placed in a round-bottom flask which was heated in an oil bath at 170° for 2 hours. Water distilled out of the mixture during the early stages of the reaction. The molten product was poured into 200 cubic centimeters of

water, and when crystallization was completed it was recrystallized from 150 cubic centimeters of water. The phthalimido- β -alanine so obtained (38 grams) melted at 150–151°.

A mixture of 41 grams of phthalimido- β -alanine and 39 grams of PCl_5 was placed in a round-bottom flask equipped with a reflux condenser and drying tube, and the mixture was heated on a steam bath for 4 hours. The POCl_3 which had formed was removed by distillation under reduced pressure on a water bath, and the last traces of it were eliminated by repeated solution of the residue in dry benzene and evaporation of this solvent under reduced pressure. In order to carry out the condensation, the product was dissolved in 250 cubic centimeters of dry benzene, and 50 grams of powdered AlCl_3 were added cautiously. The mixture was then refluxed on a steam bath for 1 hour, cooled, and poured on 250 cubic centimeters of ice cold N HCl . The benzene was removed under reduced pressure, and the precipitate was filtered off and washed with water and finally recrystallized from alcohol. The yield was 45 grams, and the melting point of the product was 129–130°.

In order to remove the phthalic acid residue from the molecule, 6 grams were dissolved in 25 cubic centimeters of glacial acetic acid, and 18 cubic centimeters of concentrated HCl were added. The mixture was refluxed for 3 hours and was then concentrated to dryness under reduced pressure. Water was added, and the phthalic acid which remained insoluble was removed. The soluble portion was concentrated under reduced pressure to dryness and recrystallized from a small volume of alcohol. Older directions (393) call for the carrying out of the hydrolysis in a sealed tube, but the modification described here is more satisfactory for large-scale operation and gave comparable yields of the hydrochloride.

Phenylpantothenone. A mixture of 1.85 grams of aminoethylphenyl ketone hydrochloride and 1.50 grams of *l*- α -hydroxy- β,β -dimethylbutyrolactone¹ were dissolved in 10 cubic centimeters of absolute methanol. The solution was heated to boiling and slowly treated with 10 cubic centimeters of N NaOH in absolute methanol. The mixture was refluxed for half an hour, cooled, treated with 15 cubic centimeters of aqueous N HCl ,² concentrated under reduced pressure to about 10 cubic centimeters, and diluted with 15 cubic centimeters of water. The resulting mixture was cooled and filtered from the crystals which had

¹ This lactone is used in the commercial synthesis of pantothenic acid and therefore is readily available.

² The normality of the acid and alkali used should be exact and not approximate.

separated.³ The filtrate was extracted four times with ethyl acetate, and the extracts were dried with MgSO_4 and freed of solvent under reduced pressure. The residue which remained was taken up in 50 cubic centimeters of absolute ether, filtered, and the ether-soluble portion was shaken out with 50 cubic centimeters of cold N NaOH to remove unchanged lactone. The ether phase was immediately dried with MgSO_4 and freed of ether under reduced pressure; 400 milligrams of a liquid were obtained. When a 20 per cent solution in alcohol was stored for several days, white crystals slowly formed. These melted at 126° . Crystallization was, however, difficult to induce. The compound was moderately soluble in water and very soluble in alcohol. When it was mixed with one equivalent of *p*-nitrophenylhydrazine in alcohol solution, and water was added, a hydrazone separated. If the dilution with water was not carried out very slowly, the hydrazone formed as an oil which solidified only with difficulty. The derivative began to soften at 110° and melted at 118° .

Synthesis of β -acetylpyridine

The method of synthesizing β -acetylpyridine was first described by Strong and McElvain (394). During the preparation it must be borne in mind that no nicotinic acid should be allowed to persist as an impurity in the final product. A mixture of 151 grams (1 mole) of ethyl nicotinate, 167 grams (1.9 moles) of ethyl acetate, and 104 grams (1.55 moles) of sodium ethoxide was allowed to stand at room temperature for about 1 hour with occasional shaking. The mixture became quite warm and assumed a deep reddish-brown color. It was then refluxed 5 to 6 hours, cooled, and diluted with an equal volume of water. The unreacted esters were extracted with ether, and the remaining aqueous solution acidified with concentrated hydrochloric acid and then made slightly alkaline with sodium carbonate solution. The oily layer of ethyl nicotinoacetate and acetoacetic ester was separated, and the aqueous layer extracted twice with ether. These ether extracts were combined with the keto ester layer and, after drying with anhydrous potassium carbonate, saturated with dry hydrogen chloride. The precipitated ethyl nicotinoacetate hydrochloride after recrystallization from an alcohol-ether mixture melted at 156 – 157.5° . The yield was 115–161 grams (50–70 per cent).

³ The white needles which formed at this point melted at 192° and gave analyses which corresponded to the formula $\text{C}_{25}\text{H}_{27}\text{O}_5\text{N}$. It was obviously not the desired product. Although it was inhibitory to the growth of all bacterial species used in this work, it was not very potent and its effect was not overcome by pantothenic acid.

A solution of 42 grams of ethyl nicotinoacetate in 300 cubic centimeters of 10 per cent hydrochloric acid was refluxed for 6 hours. The resultant solution, which gave no coloration with ferric chloride, was evaporated to dryness on a steam bath, and the remaining residue recrystallized from alcohol. A yield of 27.5 grams (96 per cent) of β -acetylpyridine hydrochloride, melting point $176-177.5^\circ$, was obtained. The free base was prepared by solution of the hydrochloride in an excess of aqueous NaOH and extraction of it with chloroform. It was then dried and distilled under reduced pressure. The boiling point was $90-92^\circ$ at 5 millimeters pressure and the melting point was $13-14^\circ$. It was readily soluble in cold water.

Synthesis of α -tocopherol quinone

The method is a modification of that of Karrer and Geiger (395). Because the metabolite (α -tocopherol) is the starting point for the preparation of the analog, steps must be taken to ensure that none of it remains in the final product; otherwise interference in the biological tests may intrude.

Ten grams of synthetic *dl*- α -tocopherol (Merck) were dissolved in 200 cubic centimeters of alcohol, and a solution of 10 grams of $\text{AuCl}_3 \cdot 2\text{H}_2\text{O}$ in 30 cubic centimeters of H_2O was added. After the mixture had stood overnight the precipitated gold was filtered off and washed with ether, and to the filtrate were added 50 cubic centimeters of H_2O and 200 cubic centimeters of ether. The aqueous layer was separated, and the ether solution was washed with H_2O until it was free of gold salts. This point could be detected readily by testing the successive washings with H_2S . The ether solution was then dried with Na_2SO_4 and freed of solvent under reduced pressure. In this manner a yellow or reddish oil was obtained. It was necessary to use an excess of $\text{AuCl}_3 \cdot 2\text{H}_2\text{O}$ in order to ensure complete oxidation. For the same reason $\text{AuCl}_3 \cdot 2\text{H}_2\text{O}$ was used in preference to FeCl_3 . Because of the excess oxidant the product was contaminated with small amounts of more oxidized substances.

Synthesis of the *p*-nitrobenzyl ether of 3,5-diiodo-N-acetyltyrosine

N-acetyldiiodotyrosine was prepared by a modification of the method of Myers (396), and this was condensed with *p*-nitrobenzyl bromide as described in a published paper (128). It was essential to follow the directions for this condensation carefully, because impure products resulted when they were modified.

One hundred grams of 3,5-diiodo-*l*-tyrosine were dissolved in one liter of 2 *N* NaOH. The solution was cooled and shaken and slowly treated

with 110 cubic centimeters of acetic anhydride. The reaction was allowed to proceed at room temperature for 2 hours, and the diacetyldiiodotyrosine was then precipitated by acidification with concentrated HCl. The precipitated product was recrystallized by solution of it in one liter of hot alcohol and addition of hot water until turbidity was produced. The yield was 93 grams, and the melting point was 186–187°. The O-acetyl group was removed by warming a solution of the compound in excess 1 N NaOH on the steam bath. This warm solution was then poured into a large volume of hot dilute HCl, and 70 grams of crystals which melted at 125° were obtained from the cold mixture.

Twenty-four grams of N-acetyl-3,5-diiodo-*l*-tyrosine were dissolved in 110 cubic centimeters of 1 N NaOH. The solution was heated in a boiling-water bath, stirred vigorously, and treated with 11 grams (1 equivalent) of *p*-nitrobenzyl bromide, added in small portions during a period of 5 minutes. Heating and stirring were continued for 15 minutes, and the clear solution was then cooled to 0° for several hours. It was essential that no excess of the bromide was used, for, when this was done, a product low in iodine and high in nitrogen was obtained. As the reaction mixture cooled, the sodium salt of the desired ether precipitated. This salt was separated in a centrifuge, washed with a small amount of cold water, and recrystallized from the minimal amount of 1 N NaOH. It was then dissolved in 750 cubic centimeters of water, and the solution was extracted once with ether, freed of ether, and then acidified with HCl. The *p*-nitrobenzyl ether of N-acetyldiiodotyrosine which precipitated was filtered off, washed, and dried. Yield was 25 grams; melting point, 82–87°. It began to soften at 82° and sintered as the temperature was raised, until complete melting occurred at 87°. The *p*-nitrobenzyl ether could be recrystallized from dilute alcohol, but from this solvent it usually precipitated as an oil which crystallized only on long standing. Purification by this means did not alter the melting point.

The importance of chemically defined rations and media

If one wishes to do an experiment with antimetabolites, it is advisable and frequently imperative that the nutrients supplied to the organism are chemically defined. This is true not only because it is necessary to know accurately the concentration of the related metabolite which is being supplied but also because many constituents of natural foodstuffs, some of which are unknown, may counteract the action of the agent under investigation. Therefore, whenever possible, experiments are done on animals fed highly purified diets or with

microorganisms cultivated in media composed of chemically defined substances. Occasionally, results observed under such conditions may then be transferred rather directly to organisms in their natural environment. The use of highly purified rations and media is a means of eliminating some of the variables and of unmasking some of the interrelationships which otherwise would be undetected.

Highly purified basal ration for mice and rats

A convenient and adequate basal ration which will support good growth of weanling rats or mice was prepared as follows: 18 grams of vitamin-free casein (Labco), 76 grams of sucrose, and 5 grams of the salts shown in Table 1 were mixed thoroughly, and then 1 cubic centi-

Table 1

Inorganic salt mixture as described by Phillips and Hart (397)

	Grams
NaCl	335
K ₂ HPO ₄	750
CaHPO ₄	140
MgSO ₄	104
CaCO ₃	600
Ferric ammonium citrate hexahydrate	55
KI	1.6
MnSO ₄ · 4H ₂ O	7
ZnCl ₂	0.5
CuSO ₄ · 5H ₂ O	0.6

All constituents except CaCO₃ were mixed and ground to a fine powder in a ball mill, and then the CaCO₃ was added.

meter of a solution of water-soluble vitamins and 1 cubic centimeter of corn oil fortified with vitamins A, D, E, and K were added. The ration was mixed thoroughly immediately after the solutions of vitamins had been spread on it. This was done in order to avoid formation of lumps. The solution of water-soluble vitamins was prepared by suspension of thiamine chloride, 20 milligrams; riboflavin, 50 milligrams; calcium pantothenate, 200 milligrams; pyridoxine hydrochloride, 20 milligrams; nicotinic acid, 100 milligrams; choline chloride, 2 grams; and *meso*-inositol, 3 grams, in 80 cubic centimeters of water. When all but a few particles of riboflavin had dissolved, 20 cubic centimeters of alcohol were added and the mixture was stored in the cold in a bottle protected from the light. The fortified corn oil was made by dissolving 1 gram of vitamin A concentrate (200,000 units), 1 gram of α -tocopherol acetate, 1 gram of vitamin D concentrate (Viosterol, 10,000 units), and 10

milligrams of 2-methylnaphthoquinone in 100 cubic centimeters of corn oil. This solution was stored in the cold with precautions to exclude light and oxygen.

The highly purified ration just described will maintain good rate of growth of rats or mice. Whenever a mixture deficient in one vitamin is required, it is only necessary to omit this constituent.

Highly purified media for the cultivation of microorganisms

Many lactobacilli and non-pathogenic streptococci and staphylococci can be grown readily in a medium prepared in the following manner: 200 milligrams of cystine, 10 milligrams of guanine, 10 milligrams of xanthine, 10 milligrams of adenine, and 10 milligrams of uracil were dissolved in 200 cubic centimeters of hot water containing 0.4 cubic centimeter of concentrated HCl; 12 grams of sodium acetate, 500 milligrams of asparagine, 400 milligrams of tryptophane, 10 grams of an acid hydrolysate of vitamin-free casein, 20 grams of glucose, 1 gram of K_2HPO_4 and 1 gram of KH_2PO_4 and 300 cubic centimeters of water were heated until solution was completed. The acid hydrolysate of casein may be purchased commercially or prepared in the laboratory by hydrolysis of vitamin-free casein with 7 *N* sulfuric acid and removal of sulfate ions in the usual fashion with barium hydroxide. The two solutions of constituents just described were mixed and then 0.2 milligram of thiamine chloride, 0.1 milligram of biotin, 0.01 milligram of pteroylglutamic acid, 0.4 milligram of calcium pantothenate, 0.8 milligram of pyridoxine hydrochloride, 0.4 milligram of nicotinic acid, 0.2 milligram of pyridoxamine, 1 milligram of riboflavin, 0.2 gram of $MgSO_4 \cdot 7H_2O$, 10 milligrams of NaCl, 10 milligrams of $FeSO_4 \cdot 7H_2O$, and 10 milligrams of $MnCl_2$ were added, and the *pH* was adjusted to 7.0 with NaOH. The solution was then made to 1 liter with water and preserved by addition of a layer of toluene. It should be kept in the cold away from light. This medium is double strength so that when an experiment is performed it is diluted with an equal volume of water or of some material to be tested. Just as with the purified rations for animals so with this medium, a culture fluid deficient in any single constituent may be prepared readily by omission of one of the components.

For the cultivation of yeast and many other fungi a useful medium may be prepared as follows: 40 grams of glucose, 6 grams of $(NH_4)_2SO_4$, 4 grams of KH_2PO_4 , 200 milligrams of asparagine and 10 milligrams of inositol were dissolved in 500 cubic centimeters of water; 250 milligrams of $MgSO_4 \cdot 7H_2O$ were dissolved in water and were added to this solution. Similarly 250 milligrams of calcium chloride were dissolved

in water and added. One milligram of calcium pantothenate, 0.1 milligram of pyridoxine hydrochloride, 0.02 milligram of biotin, 0.5 milligram of thiamine chloride, 1 milligram of ZnSO_4 , 1 milligram of MnCl_2 , 0.5 milligram of FeCl_3 , 1 milligram of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, and 1 milligram of KI were added, and the volume was adjusted to 1 liter. This medium is double strength. A variety of such mixtures has been used for the cultivation of yeasts and other fungi, but this one serves well for those species which do not demand preformed amino acids or vitamins beyond those included. Many common species will grow luxuriantly in the medium just described.

Competition of *p*-aminobenzoic acid and sulfanilamide in the growth of bacteria

This classical demonstration has been made with many kinds of microorganisms and in a variety of media. The phenomenon can be observed with *Lactobacillus arabinosus* cultivated in the medium described. It is advisable to omit the pteroylglutamic acid. Graded concentrations of sulfadiazine ranging from 1 gamma per cubic centimeter to 100 gammas per cubic centimeter were tested in the presence of several different concentrations of *p*-aminobenzoic acid. The amounts of this metabolite were 0.001 gamma per cubic centimeter, 0.01 gamma per cubic centimeter, and 0.1 gamma per cubic centimeter. The inoculum was grown, washed, and diluted as in the succeeding experiments. The organisms were cultured in 10-cubic centimeter portions of medium contained in test tubes and were incubated at 30° for 2 days. Turbidity was then measured photometrically, and for each level of *p*-aminobenzoic acid a curve was constructed which related turbidity to concentration of the analog. From these curves the amount needed to reduce growth to half maximum was calculated, and, when this was done, the competition between metabolite and analog was readily discernible.

Production of thiamine deficiency of mice with pyriethamine

Weanling mice approximately 21 days old were caged individually in screen-bottomed jars and fed a ration such as that just described except that the thiamine content was reduced one-half, in order to avoid an excess and yet to provide enough. Each mouse was given orally 0.1 milligram of pyriethamine each day. This was conveniently administered as 0.01 cubic centimeter of an aqueous solution. The characteristic signs of thiamine deficiency developed during the second week of the experiment. Although daily dosing was carried out, the whole train of manifestations of disease was also called forth merely

by feeding 1 milligram of the compound on the first day of the experiment. Six to 9 days after this, the characteristic signs of the deficiency appeared, and these have been described in Chapter 1.

Competition of thiamine and pyrithiamine in mice

The conditions of the previous experiment were maintained except that thiamine was omitted from the basal ration, and each day the mice were given orally a solution containing thiamine and pyrithiamine. The amounts were varied from 1.5 gammas of the vitamin to 60 gammas of it. With each level, graded amounts of pyrithiamine were included. The data shown in Table 2 of Chapter 1 illustrate the responses of the animals. It was seen that the toxicity of pyrithiamine was not an absolute value but rather was directly proportional to the amount of thiamine which the animals were receiving.

Striking cures of the advanced signs of the deficiency were produced in the following manner. Mice were given a single dose of 1 milligram each of pyrithiamine as was described in the preceding section. When the manifestations of disease were far advanced, a single oral dose of 1 milligram of thiamine was given. Improvement was noticeable within 1 hour, and cure seemed complete in 24 hours.

Selective reversible inhibition of microbial growth with pyrithiamine

To show the selective reversible inhibition of microbial growth with pyrithiamine the yeast *Endomyces vernalis* served well. The medium described in the preceding section was used except that thiamine was omitted. The organisms were cultured in 10-cubic centimeter aliquots of the medium contained in 50-cubic centimeter Erlenmeyer flasks and were grown at 37° for 24 hours. Thiamine and pyrithiamine were each sterilized separately by filtration of their aqueous solutions in order to avoid destruction of them by heat. The remaining basal medium was sterilized in an autoclave at 120°. A series of flasks was fortified with 0.01 gamma of thiamine per cubic centimeter of medium, and a second series with 0.02 gamma. A third series was made to contain 0.1 gamma, and a fourth 1.0 gamma of the vitamin. In each series, graded amounts of pyrithiamine, designed to cover the range of 0.2–20 gammas of pyrithiamine per cubic centimeter, were tested. The inoculum for each flask was prepared by collecting the cells from 1 cubic centimeter of a vigorously growing culture by centrifugation. These were then washed three times with sterile water and finally suspended in 100 cubic centimeters of sterile water. This procedure was necessary in order to minimize transfer of thiamine in the inoculum. One drop of the diluted, washed suspension was used to seed

each flask. After the growth period of 24 hours, the turbidity of the contents of each flask was determined photometrically. For each series containing the same quantity of thiamine, the amount of pyrithiamine which just permitted half maximal multiplication was determined by plotting graphically turbidity against dosage of analog. Interpolation on this curve then showed that when 0.01 gamma of thiamine per cubic centimeter was used, 0.33 gamma per cubic centimeter of pyrithiamine was required. When 0.02 gamma of thiamine was present, 0.65 gamma of pyrithiamine was needed, and when 0.1 gamma of the thiamine had been added, 3.3 gammas of the analog were necessary.

The preceding experiment showed the competition between thiamine and pyrithiamine in the growth of a yeast. The selective effect of this analog was demonstrated as follows. A variety of microorganisms such as that shown in Table 3 of Chapter 1 was assembled and each was grown in one of the highly purified basal media described in this chapter. The bacteria were cultivated in the first, and the fungi in the second of these. Thiamine was reduced so that 0.01 gamma per cubic centimeter was present in the final media. Graded amounts of pyrithiamine ranging from 0.1 gamma per cubic centimeter to 1 milligram per cubic centimeter were added, and inoculation and incubation and the reading of the results were carried out as in the experiments with *E. vernalis*.

In order to determine the nutritional requirements for thiamine each species was inoculated into the proper basal medium minus this vitamin. The ability to grow with and without thiamine was then compared.

The results of these experiments showed that only those species were inhibited in growth by pyrithiamine which exhibited a nutritional requirement for thiamine or its constituent parts.

Competition between pantothenic acid and phenylpantothenone, and the dependence of reversal on nutritional requirement

For this experiment the basal media minus calcium pantothenate were used. The organisms were *Lactobacillus casei*, *Lactobacillus arabinosus*, *Endomyces vernalis*, and *Saccharomyces cerevisiae*. The last two organisms were cultivated in the synthetic medium for yeast and fungi. The general procedure was similar to that described in the preceding section except that the vitamin and analog in this case were added to the basal media before sterilization by autoclaving. Since phenylpantothenone was not readily soluble in water, solutions of the desired concentration were prepared by adding a 10 per cent alcoholic solution to water. In this way it was made to remain soluble,

whereas the solid material could not readily be taken up in water. The bacteria were cultured in test tubes and the yeasts in Erlenmeyer flasks. *S. cerevisiae* and *L. arabinosus* were incubated at 30°, and the others at 37°. To each container 0.04 gamma of calcium pantothenate per cubic centimeter of medium was added. After inoculation and incubation turbidities were measured photometrically and dose-response curves were constructed. These showed that half maximal inhibition of growth resulted with *L. casei* when 28 gammas of phenylpantothenone per cubic centimeter were present; with *L. arabinosus*, 180 gammas; with *S. cerevisiae*, 33 gammas; and with *E. vernalis*, 39 gammas.

When each of these organisms was grown in the appropriate basal medium minus pantothenic acid, the first three were found to depend on this vitamin, whereas the last one grew well without it. However, for *S. cerevisiae* pantothenic acid was not required, provided that β -alanine was present in the medium.

The ability of calcium pantothenate to overcome the toxicity of phenylpantothenone was determined with each species by addition of ten times as much of the vitamin as was used in the preceding trials; ready reversal was observed in the case of *L. casei* and *L. arabinosus*. In fact, with a tenfold increase in the vitamin, a corresponding increase in the analog was needed to cause retardation of growth. The other two organisms were not influenced in this respect by increases in the amount of the vitamin. Thus the reversal by pantothenic acid of the toxicity of phenylpantothenone was correlated with the nutritional need of the organisms for pantothenic acid. More extensive trials with other species confirmed this conclusion and illustrated that the difference just described was not a function of the dissimilarity of the two basal media. Thus, with *Escherichia coli* grown in the medium employed for bacteria the toxic action of phenylpantothenone was not overcome by increases in the amount of calcium pantothenate. This bacterium, like the yeasts, did not require pantothenic acid as a growth factor.

Production of nicotinic acid deficiency of mice with β -acetylpyridine as an example of prior fixation of the analog and exclusion of the metabolite

For these experiments weanling mice were fed the highly purified basal ration minus nicotinic acid. Under these conditions mice do not require this vitamin in the diet, because they make their own supplies from the tryptophane contained in the casein. The animals were housed individually in cages with screen bottoms, and each was given

β -acetylpyridine orally each day. For each dose level at least 6 animals were used. Amounts of β -acetylpyridine greater than 2 milligrams per day were injurious, and 4 milligrams per day were sufficient to kill practically all individuals within 2 weeks. The signs of toxicity appeared within a few hours of administration of the analog. However, the fiery red appearance of the margins of the tongue which is frequently seen in dietary deficiency of nicotinic acid developed slowly, so that animals which did not survive for more than a few days did not exhibit this sign. When nicotinic acid or its amide or ethyl ester was administered after the ingestion of 4 milligrams of β -acetylpyridine, no protection of the mice from the effects of the analog was observed. This was true even when large amounts of the vitamin were given. However, if nicotinic acid or nicotinamide was added to the basal ration and this was fed to the mice for 2 days before the administration of the analog was begun, a competitive antagonism was demonstrated. Thus, the addition of 200 milligrams of the vitamin per 100 grams of ration led to complete protection against 4 milligrams of β -acetylpyridine per day; 10 milligrams of the analog per day showed some toxicity under these conditions, but this toxicity was eradicated by increasing the amount of nicotinic acid to 500 milligrams per 100 grams of ration.

Selective action of α -tocopherol quinone on pregnant mice

To show the selective action of α -tocopherol quinone on pregnant mice, the basal ration for mice was modified slightly. The fortified corn oil was omitted, and to every 100 parts of the mixture were added 5 parts each of yeast, lard, and cod liver oil. The amount of sucrose was reduced to provide room for these new components. Adult female mice were taken for the experiments during the first 4 days of the gestation period. Each day 100 milligrams of α -tocopherol quinone were given orally to each animal. The mice were caged individually in metal boxes provided with a bedding of wood shavings. At about the fourteenth day of pregnancy some of the animals developed hemorrhage from the reproductive tract. Very occasionally this proved to be severe enough to cause death. All individuals were continued on the experiment until 4 weeks had elapsed from the beginning. Less than 10 per cent bore litters, and among those which did the number of young per litter averaged 3. This was in contrast to the behavior of untreated females or of those which had received α -tocopherol quinone and in addition small amounts of vitamin K. With these all bore litters which averaged 7 individuals each.

After the termination of the test period with α -tocopherol quinone the mice were returned to a normal stock ration and were mated once again. After the usual period of gestation (21 days) normal litters were born. It was thus clear that no permanent damage had been done.

When non-pregnant females were subjected to the same treatment as that described above, no detectable signs of toxicity of α -tocopherol quinone were found. This was true even when the amount of the analog was doubled. It was, therefore, clear that the compound had exerted a selective action on the pregnant individuals.

Among the treated mice several were autopsied on the sixteenth day of gestation and several more on the twentieth day. These examinations revealed that death of the embryos had occurred and that resorption was in progress. The resorption was more noticeable on the animals taken on the twentieth day.

The experimental conditions were modified in order to carry out the demonstration with somewhat smaller doses of the analog, and also to show that pregnancy could be established while it was being administered. Thus, when a single dose of 200 milligrams of α -tocopherol quinone was injected intraperitoneally, the results were quite similar to those found when daily oral doses were given. Similarly, when the oral treatment was begun 1 day prior to mating, pregnancy was established and interrupted in the same fashion as that described under the conditions used previously.

Nullification of the toxic effects of excess thyroxine with the *p*-nitrobenzyl ether of diiodotyrosine

The demonstration was based on the fact that thyroxine is a lethal agent to tadpoles. As is well known, minute quantities of this hormone stimulate markedly the rate of metamorphosis of these animals. Larger amounts cause rapid metamorphosis which is abruptly terminated by death. For the purposes at hand, protection against death proved to be a better criterion of antithyroxine activity than measurements of the rate of change into frogs. Furthermore, the aim of the work was to find agents which would protect against the toxic effects of excess thyroxine.

Six groups of 12 tadpoles, each animal 3 to 4 centimeters in total length and in the stage of development just prior to the appearance of hind leg buds, were immersed in 500-cubic centimeter portions of a 1:20 dilution of frog Ringer's solution in distilled water. Tap water could be used in place of this diluted salt solution, provided that it had not been chlorinated excessively.

Solutions of thyroxine and of the *p*-nitrobenzyl ether of N-acetyl-diiodotyrosine were prepared by addition of one equivalent of 0.1 *N* NaOH to the dry substance, and when it had dissolved completely the pH was adjusted to 7. The first two groups of tadpoles were used as controls and hence were unsupplemented. To each of the next two was added a solution of 1 milligram of *dl*-thyroxine. Each of the third pair was treated with 1 milligram of *dl*-thyroxine and 375 milligrams of the *p*-nitrobenzyl ether of N-acetyl-3,5-diiodotyrosine. After the animals had remained in these solutions for 24 hours they were strained out of the liquid, washed gently, and placed in fresh 1-liter portions of diluted Ringer's solution and fed with a small piece of hard-boiled egg white. Each day the animals were transferred to fresh diluted Ringer's solution and fed. They were observed carefully each day for a period of 2 weeks in order to determine whether accelerated metamorphosis was in progress and whether toxic effects were developing.

At the end of the 2-week test period all the animals in the first two groups were alive and showed no detectable progression of metamorphosis. Those which had received thyroxine alone were dead. They had shown markedly accelerated rates of metamorphosis, had progressed through the stages of development of hind legs and usually of forelegs, and had exhibited a characteristic resorption of the tail and the appearance of angular segments of the head and body which gave them a frog-like aspect. In contrast the tadpoles which had received thyroxine plus the analog remained alive. Some accelerated metamorphosis did occur, and this was exhibited by the development of hind-leg buds or even of hind legs in some individuals. However, the rate of change was not comparable to that seen in the preceding group. By alteration of the concentration of both thyroxine and the analog to which the animals were exposed on the first day of the experiment, a competition between hormone and its structural relative was observed similar to that which was found in the preceding experiments with bacteria and mice. The results of such experiments have been summarized in Table 1 of Chapter 7. The range of concentration was somewhat limited, because as the amount of dissolved material was increased the osmotic pressure of the solutions became unfavorable to the animals.

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